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13. ABSTRACT *(Maximum 200 Words)*

The toxicity of chemically reactive oxygen species (ROS) is thought to make a significant contribution to the death of nerve cells caused by many neurotoxins as well as in stroke and Parkinson's disease. During all of these events, some groups of nerve cells are spared relative to others. It is therefore likely that biochemical mechanisms exist which lead to increased resistance to oxidative stress and other forms of cytotoxicity. It was the goal of this proposal to understand how nerve cells defend themselves against neurotoxins that kill cells via ROS and oxidative stress. To accomplish this goal, we have studied toxin sensitive cell death pathways and have selected groups of cells which are very resistant to ROS generated in a model system which mimics some aspects of acute neurotoxicity and stroke. It was shown that the translation factor eIF2α mediates cell death involving oxidative stress, while the classical pro-apoptosis factor Bax is not involved. The activation of soluble guanylate cyclase is also required for cell death, for dopamine D4 receptor activation inhibits cGMP production and blocks cell death. Other inhibitors of oxidative stress induced nerve cell death were also discovered. These include a unique group of plant flavanoids and the activation of the transcription factor HIF-1. Finally, it was shown that while there is a great deal of cross-resistance to many neurotoxins, the components of the cell death pathways are sometimes distinct.

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INTRODUCTION

The accumulation of oxidatively damaged macromolecules results from the exposure to several chemical warfare agents and is thought to contribute heavily toward the genesis of numerous diseases of old age. To date, a great deal is known about the causes of oxidative stress, but very little about how cellular metabolism is altered to successfully cope with this condition. If it were possible to understand at the molecular level how cells are able to cope with increases in oxidative stress, then it should be possible to artificially modify these defense mechanisms to inhibit or slow the damage. The research accomplishments outlined below help to define the molecular mechanisms by which cells become resistant to oxidative stress and also identify a number of conditions which block nerve cell death pathways. This knowledge may be used to slow down or prevent oxidative damage associated with exposure to neurotoxins as well as those associated with the diseases of aging. The information should have a significant impact upon the treatment of neurotoxicity and environmental conditions associated with oxidative stress.

BODY

As outlined in the abstract, several areas of work have been completed during the last three years. These include examining the role of the cell death (apoptosis) gene in oxidative glutamate toxicity and excitotoxicity, the role of eIF2α in oxidative stress, the involvement of oxidative glutamate toxicity in excitotoxicity, the role of soluble guanylate cyclase in neuroprotection via the activation of dopamine receptors, the neuroprotective mechanisms of flavonoids, the overlap of neuroprotective pathways, and finally, the role of the transcription factor HIF-1 in neuroprotection. The results from each of these studies will be outlined below and the completed manuscripts with all of the data and details are affixed to the appendix.

A. Bax

Bax is a required protein for most forms of apoptotic programmed cell death. It is thought to regulate the permeability of mitochondria to proteins which mediate the activation of caspases and other components of apoptosis. Nerve cell death from both oxidative glutamate toxicity and excitotoxicity share a few characteristics with classical apoptosis, but no one has ever examined the role of Bax, the major player in the classical apoptosis pathway, in these pathways. This is critical information, for if Bax is not involved, then another form of programmed cell death than classical apoptosis must take place during glutamate neurotoxicity. To approach this issue, we used mice which lack the Bax gene, so called Bax knock-out mice. These mice are developmentally abnormal and die before birth, but it is possible to obtain brain nerve cells for primary cultures from E14 embryos and study their response to glutamate. When this was done, it was shown that cortical neurons died equally well when they were isolated from homozygous or heterozygous Bax deletions and from wild type mice. In contrast, the rate of spontaneous cell death when cells are initially placed in culture, due to growth factor withdrawal, was greatly retarded in the cell cultures from Bax knock-out animals. These results clearly show that Bax is not involved in either oxidative glutamate toxicity or excitotoxicity caused by a brief exposure to low concentrations of glutamate. They therefore strongly suggest that a unique form of nerve cell death is involved in glutamate toxicity, which certainly utilizes a distinct set of molecular components.

B. eIF2α and oxidative stress

Although programmed cell death (PCD) is a widely used mechanism for sculpturing the developing nervous system, its inappropriate activation leads to premature nerve cell death in neuropathological disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). These forms of nerve cell death as well as those caused by a wide variety of neurotoxins are thought to be linked to oxidative stress, for antioxidant systems are upregulated and there is

extensive evidence for excessive lipid and protein peroxidation. Associated with oxidative stress, there is usually an early and highly specific decrease in neuronal glutathione content. In the substantia nigra of PD patients, this loss may precede the death of dopaminergic neurons. In addition, the inhibition of γ-glutamyl-cysteine synthetase (γGCS), the rate limiting step in GSH synthesis, results in the selective degeneration of dopaminergic neurons, and also potentiates the toxicity of 6 hydroxydopamine, MPTP and MPP^+. These data suggest that GSH and oxidative stress play pivotal roles in neurotoxicity and in the pathogenesis of AD and PD.

There are several ways in which the concentration of intracellular GSH and the oxidative burden of cells can be regulated. One of these is through extracellular glutamate. Although glutamate is generally thought of as both a neurotransmitter and an excitotoxin, extracellular glutamate can also kill neurons through a non-receptor mediated pathway which involves the glutamate-cystine antiporter, system Xc^-. Under normal circumstances the concentration of extracellular cystine is high relative to intracellular cystine, and cystine is imported via the Xc^- antiporter in exchange for intracellular glutamate. Cystine is ultimately converted to cysteine and utilized for protein synthesis and to make the antioxidant glutathione (GSH). However, when there is a high concentration of extracellular glutamate, the exchange of glutamate for cystine is inhibited, and the cell becomes depleted of cysteine and GSH, resulting in severe oxidative stress. The cell eventually dies via a series of events which include the depletion of GSH, a requirement for macromolecular synthesis and caspase activity, lipoxygenase (LOX) activation, soluble guanylate cyclase activation, reactive oxygen species (ROS) accumulation, and finally Ca^{2+} influx.

Programmed cell death caused by oxidative glutamate toxicity has characteristics of both apoptosis and necrosis, and has been well studied in primary neuronal cell cultures, neuronal cell lines, tissue slices, and in the immortalized mouse hippocampal cell line, HT22. HT22 cells lack ionotropic glutamate receptors but die within 24 hours after exposure to 1-5 mM glutamate. Although the biochemical events have been well studied, little has been done to identify the transcriptional/translational changes which contribute to the glutamate-induced pathway of programmed cell death. Changes in gene expression clearly play a role in the cell death cascade since macromolecular synthesis is required early in the death pathway.

Using an experimental nerve cell model for oxidative stress and an expression cloning strategy, a gene involved in oxidative stress-induced programmed cell death was identified which both mediates the cell death program and regulates GSH levels. Two stress-resistant clones were isolated which contain antisense gene fragments of the translation initiation factor eIF2α and express a low amount of eIF2α. Sensitivity is restored when the clones are transfected with full length eIF2α; transfection of wild-type cells with the truncated eIF2α gene confers resistance. The phosphorylation of eIF2α also results in resistance to oxidative stress. In wild-type cells oxidative stress results in rapid glutathione depletion, a large increase in peroxide levels, and an influx of Ca^{2+}. In contrast, the resistant clones maintain high glutathione levels and show no elevation in peroxides or Ca^{2+} when stressed, and the glutathione synthetic enzyme gamma-glutamyl cysteine synthetase (γGCS) is elevated. The change in γGCS is regulated by a translational mechanism. eIF2α is therefore a critical regulatory factor in the response of nerve cells to oxidative stress and in the control of the major intracellular antioxidant, GSH, and may play a central role in the many neurodegenerative diseases and toxicities associated with oxidative stress.

C. Oxidative glutamate toxicity and excitotoxicity

The physiological consequences of extracellular glutamate are mediated by three classes of membrane proteins within the central nervous system (CNS). These are ionotropic glutamate receptors, metabotropic glutamate receptors, and the cystine/glutamate antiporter. Ionotropic glutamate receptors have two known roles. They are responsible for the majority of excitatory

neurotransmission within the CNS and also for a great deal of CNS pathology. In cases of stroke or trauma, excessive extracellular glutamate leads to nerve cell death via the activation of NMDA receptors. This phenomenon, which can be reproduced in cell culture is termed excitotoxicity. In contrast to ionotropic glutamate receptors, the metabotropic glutamate receptors (mGluRs) are G-protein coupled membrane proteins with a wide variety of biological functions. As described above, a third target for extracellular glutamate in the CNS is the inhibition of the glutamate/cystine antiporter x_c^- which results in a form of oxidative stress and cell death called oxidative glutamate toxicity. The glutamate/cystine antiporter couples the import of cystine to the export of glutamate. Concentrations of extracellular glutamate as low as 100μM, which is well below the level of extracellular glutamate found in models of stroke and trauma, completely inhibit the uptake of cystine. Cystine is required for the synthesis of the potent intracellular reducing agent glutathione (GSH). When GSH is depleted by extracellular glutamate, cells die from a form of programmed cell death.

The potential role of oxidative glutamate toxicity in ischemia and trauma is not understood, but there have been strong indications that several cell death pathways are involved in the excitotoxicity cascade. In localized cerebral infarction, the neurons in the epicenter die rapidly, while those more distal remain viable for several hours. Multiple forms of nerve cell death have also been identified in excitotoxic CNS primary culture paradigms following exposure to glutamate. In primary cultures of cerebellar granule cells exposed to glutamate, there is a rapid necrotic phase, followed by delayed apoptotic-like cell death. During oxygen-glucose deprivation of primary mouse cortical cultures or organotypic cultures of the rat hippocampus, some cell death occurs from non-ionotropic receptor-mediated mechanisms. All of these observations are consistent with in vivo data which show that non-receptor mediated programmed cell death may occur following ischemic insults. In addition, a number of parameters change dramatically during CNS stress which lead to the observed high exogenous glutamate. These include the direct release of glutamate from cells, the enzymatic conversion of glutamine to glutamate, and the shut down of nerve and glial glutamate uptake systems by pro-oxidant conditions. It is therefore of interest to determine if oxidative glutamate toxicity can play a significant role in nerve cell death associated with the excitotoxicity cascade.

In the manuscript attached to the appendix, we show that a portion of the cell death associated with NMDA receptor initiated excitotoxicity can be caused by oxidative glutamate toxicity. In primary mouse cortical neurons cell death resulting from the short term application of 10μM glutamate can be divided into NMDA and non-NMDA receptor dependent phases. The non-NMDA receptor dependent component is associated with high extracellular glutamate and is inhibited by a variety of reagents which uniquely block oxidative glutamate toxicity. These include metabotropic glutamate receptor agonists, antioxidants, and a caspase inhibitors. In addition, it is shown that the concentration of extracellular glutamate rises to several hundred micromolar, probably due to the conversion of glutamine to glutamate in the culture medium (glutamine is equally high in CNS tissue) by the enzyme glutaminase released from lysed cells. These results suggest that oxidative glutamate toxicity toward neurons lacking functional NMDA receptors can be a component of the excitotoxicity initiated cell death pathway.

D. Dopamine and D4 Receptors

The protective effects of dopamine, apomorphine and apocodeine, but not epinephrine and norepinephrine, are antagonized by dopamine D4 antagonists. A dopamine D4 agonist also protects and this protective effect is inhibited by U101958, a dopamine D4 antagonist. Although the protective effects of some of the catecholamines are correlated with their antioxidant activities, there is no correlation between the protective and antioxidant activities of several other ligands. Normally glutamate causes an increase in reactive oxygen species (ROS) and intracellular Ca^{2+}. Apomorphine partially inhibits glutamate-induced ROS production and

blocks the opening of cGMP-operated Ca^{2+} channels which lead to Ca^{2+} elevation in the late part of the cell death pathway. These data suggest that the protective effects of apomorphine on oxidative stress-induced cell death are mediated by dopamine D4 receptors via the regulation of cGMP-operated Ca^{2+} channels.

E. Flavonoids

Flavonoids are a group of several hundred diphenylpropanes which are widely distributed in plants and are generally thought to be beneficial dietary supplements, perhaps working as antioxidants. Since we have previously shown that several aromatic antioxidants are able to protect nerve cells from oxidative stress induced toxicity. The goal of this research is to identify potent neuroprotective molecules which may have clinical use. Because flavonoids are relatively non-toxic natural products, they may be of use in this regard. Toward this end we screened a large number of flavonoids for their protective effect against glutamate-induced toxicity and five other forms of oxidative stress to HT22 hippocampal neurons as well as primary rat cortical neurons. In addition, it was asked where in the glutamate-induced cell death program the individual flavonoid acts. Many but not all of the tested flavonoids protect cells from the various forms of oxidative stress. Three structural requirements of flavonoids for protection are the hydroxylated C3, an unsaturated C ring, and hydrophobicity. We also found three distinct mechanisms of protection. These include increasing intracellular GSH, directly lowering levels of ROS, and preventing the influx of Ca2+ despite high levels of ROS. In addition, individual flavonoids can protect by more than one mechanism. For example quercetin and fisetin alter GSH metabolism and act as antioxidants at the same time. Baicalein and luteolin can act as lipoxygenase inhibitors but they can also act as antioxidants. Finally, some flavonoids may protect the cells from glutamate by directly inhibiting ROS production by mitochondria, as well as being antioxidants. These data show that the mechanism of protection from oxidative insults by flavonoids is highly specific for each compound. Since cellular oxidative stress is an important factor in various diseases, including arteriosclerosis, ischemia, trauma, Alzheimer's disease, Parkinson's disease, and AIDS as well as aging itself, flavonoids may have multiple beneficial effects in the treatment of these conditions.

F. Commonality of Cell Death Programs

A goal of this proposal is to understand the mechanisms involved in nerve cell death in response to toxins and to determine to what extent the toxic response pathways are shared between toxins. Perhaps more importantly it is necessary to determine if there is any overlap with respect to the protective resistance mechanisms used by nerve cells to protect themselves from one toxin or another. To this end, two sets of experiments were done. In the first, a clonal cell line was selected which is very resistant to glutamate or amyloid Aβ. It was then asked if these cell lines are more or less resistant to other toxins. In the second set of experiments, we challenged the HT22 cell line with a variety of toxins, and then determined if reagents known to protect cells from glutamate also protected against the other toxins.

By growing HT22 hippocampal or PC12 nerve cells in high concentrations of glutamate or Aβ for extended periods of time it is possible to select for clones which are resistant to glutamate or Aβ. One each of these resistant clones was used to determine if cells resistant to the form of oxidative stress were also resistant to other forms of toxicity. Cells resistant to glutamate or Aβ toxicity were generally resistant to other types of toxic insults. These include BSO, an inhibitor of GSH synthesis which depletes GSH stores and the reduction of exogenous cystine, which also depletes GSH. Glutamate resistant cells are about 5-fold more resistant to glucose starvation, a condition which occurs when cells are starved for blood in conditions of ischemia and stroke. The HT22r2 line is also about 10-fold more resistant to hydrogen peroxide and arsenite. Arsenite is toxic via an ill-defined mechanism, but has been shown to cause increases in ROS production which damage DNA. Rotenone also is toxic via an increase in ROS production, and

is thought to be the cause of some forms of Parkinson's disease and is used to generate a mouse model for the disease. HT22r2 cells are more resistant to rotenone than wild type, but the unusual shape of the dose-response curves makes it difficult to quantitate. Finally, cisplatin causes DNA damage in much the same way as nitrogen mustards. Glutamate resistant cells are not significantly more resistant to cisplatin than their parental cells.

Since a fair amount is known about how HT22r2 cells become resistant to glutamate, some conclusions can be made regarding the common themes which can protect cells from the various toxic insults. In glutamate resistant cells the expression levels of neither heat shock proteins nor apoptosis-related proteins are changed in the resistant cells. In contrast, the antioxidant enzyme catalase, but not glutathione peroxidase nor superoxide dismutase, is more highly expressed in the resistant than in the parental cells. In addition, the resistant cells have enhanced rates of GSH regeneration due to higher activities of the GSH metabolic enzymes γ-glutamylcysteine synthetase and GSH reductase. GSH S-transferases activities are also elevated. As a consequence of these alterations, the glutamate resistant cells are also more resistant to organic hydroperoxides and anticancer drugs that affect these GSH enzymes. It is therefore very likely that GSH plays a central role in protecting cells from multiple forms of toxicity, including arsenite and glucose starvation.

The alternative approach to examining the mechanistic interaction between cell death pathways elicited by different neurotoxins is to treat cells with the different toxins and then ask if reagents which protect cells from glutamate toxicity also protect from arsenite, cisplatin, etc. Glutamate, BSO, and hydrogen peroxide toxicity, as well as cystine deprivation are usually rescued by a common set of reagents inhibit oxidative glutamate toxicity. Some of these reagents also partially rescue cells from glucose starvation, but most do not alter arsenite and cisplatin toxicity. The glutamate resistant cells are more resistant to arsenite toxicity, but none of the reagents which block glutamate toxicity clearly block arsenite toxicity. The reason for this is not clear. It can be concluded, however, that regents which block glutamate toxicity in general do not inhibit other forms of cell death caused by pro-oxidant conditions.

G. The Transcription HIF-1 and Neuroprotection

The major goal of this proposal is to identify conditions or reagents which protect nerve cells from toxins which kill via pro-oxidant pathways. We have shown previously that both oxidative glutamate toxicity and Aβ kill cells by this mechanism. We also have shown a number of years ago that Aβ-induced cell death can be inhibited by iron chelators such as mimosine (MIM) and desferrioxamine (DFO). It has recently been shown by others that iron chelators prevent most of the pathological features of AD in mouse models. It is known, however, that the iron chelators MIM and DFO induce the transcription factor hypoxia inducible factor 1 (HIF-1) in addition to chelating iron. We have recently shown in a manuscript in preparation, summarized in the following paragraphs, that iron chelators protect nerve cells from neurotoxins by a HIF-1 dependent mechanism which is independent of iron chelation. Since DFO is an approved drug for other indications, these results may present a unique opportunity to protect the nervous system from some neurotoxin exposures. The following paragraphs summarize the evidence.

When 50 to 100 μM DFO or MIM are added to cultures of the hippocampal nerve cell line HT22, the sympathetic nerve-like cell line PC12, or the CNS rat neural cell line B12 there is a rapid induction of DNA binding by HIF-1 as defined by band-shift assays, reaching a maximum in about 4 hrs, then decreasing slightly after 21 hrs. Concomitantly, there is a protection from Aβ toxicity in the PC12 and B12 cell lines, and a protection from glutamate toxicity in the neuronal HT22 cells. The band shift assays were confirmed by "super shift" assays using an antibody against HIF-1. It was shown on Westerns that HIF-1 protein levels increase upon exposure to the iron chelators, and Northern blots showed that the HIF-1 nRNA also increases.

Therefore the induction of the HIF-1 transcription factor by iron chelators is highly correlated with resistance to two forms of neurotoxin-induced cell death. Is it sufficient?
To separate the iron chelation mechanism of MIM and DFO from HIF-1 with regard to neuroprotection, a plasmid construct was made in which its mRNA is not rapidly degraded as is the (unstable) mRNA from the wild type HIF-1 gene. This construct was then transfected into B12 and HT22 cells, and their resistance to oxidative stress-induced cell death assayed. It was shown that both cell lines are much more resistant than cells expressing the empty plasmid. It can be concluded that the expression of HIF-1 alone is sufficient to impart neuroprotection.

As outlined in the earlier sections of this summary statement, we have isolated cell lines which are resistant to Aβ and glutamate toxicity. Do these cells have elevated levels of HIF-1? The data show that both PC12 and HT22 resistant cells have slightly elevated levels of HIF-1. Since these cells are very resistant to many neurotoxins, HIF-1 seems to play a role in global defense mechanisms against many forms of stress.

Finally, the activation of glial cells by toxins such as Aβ leads to the secretion of toxic cytokines as well as pro-oxidants such as superoxide. Glial activation is associated with most chronic neurodegenerative diseases, such as AD and Parkinson's. We have shown that exposure of glial cells to MIM or DFO prior to the addition of Aβ, which activates glial cells, prevents glial activation, and that this effect is highly correlated with the DNA binding of HIF-1 in gel-shift assays. It follows that the induction of HIF-1 activity by iron chelators facilitates neuroprotection both directly and indirectly by blocking glial activation which can severely stress or kill neurons.

KEY RESEARCH ACCOMPLISHMENTS

- The Bax gene product is not involved in oxidative glutamate toxicity or excitotoxicity.
- The translation initiation factor eIF2α can serve as a switch which determines whether nerve cells live or die during oxidative stress. The eIF2α functions by regulating the level of intracellular glutathione by determining the level of the glutathione synthetic enzyme, γGCS.
- The unique programmed cell death pathway, oxidative glutamate toxicity, is a component of the widely studied but little understood excitotoxicity cascade, which is involved in many forms of oxidative stress induced by trauma and disease.
- The activation of dopamine D4 receptors protects cells from oxidative stress induced cell death.
- Flavonoids and related compounds inhibit neurotoxicity by three distinct mechanisms: enhancing GSH levels, acting as antioxidants, blocking cGMP induced calcium entry.
- Cells selected for resistance to one neurotoxin (glutamate) are resistant to some other neurotoxins.
- Reagents which protect cells from glutamate toxicity also protect cells from peroxide toxicity, but not from arsenite and cisplatin toxicity.
- The activation of the transcription factor HIF-1 was shown to protect nerve cells from several forms of oxidative stress. This is an important, clinically relevant, observation since the synthesis of HIF-1 can be initiated by clinically approved drugs such as iron chelators.

REPORTABLE OUTCOMES

- Eight manuscripts (in appendix), and one in preparation
- One Ph.D. student (Shirlee Tan) who worked on eIF2α graduated and now works for the EPA
- Research experience for a pre-med student (Dana Piasecki) working on this project
- Research experience for post-doctoral students, T. Soucek, Y. Sagara, and K. Ishigi

CONCLUSIONS

During the past three years we have studied one of the major nerve cell death pathways activated in response to various forms of oxidative stress and characterized some of the intermediate steps. More importantly, we have identified a number of reagents and conditions which specifically block the cell death pathway. These neuroprotective agents include dopamine analogues which activate the dopamine D4 receptor, a small class of flavonoids, conditions which cause the phosphorylation of the translation initiation factor eIF2α, and the activation of the transcription factor HIF-1. This work resulted in six published manuscripts in very good journals, one manuscript in preparation, and two review articles.

On the basis of this information it may then be possible to artificially activate the HIF-1 transcription factors or shut off the activity of eIF2α and therefore express the set of proteins involved in the resistance to oxidative stress. For example, HIF-1 mediates the induction of a battery of genes involved in neuroprotection. Some of these genes are upregulated in the resistant cells described by our laboratory. Since HIF-1 can be induced pharmacologically by iron chelators, it may be possible to induce the necessary antioxidant genes before or immediately after exposure to neurotoxins, leading to protection. Clearly more sophisticated methods will become available and can be easily tested in the experimental system described here. In addition, we have identified a unique set of flavonoids which are neuroprotective, and these should be studied in much greater detail because of their unique modes of action and because, as beneficial natural products, they are not toxic and could immediately be put into clinical trials. The same argument could be made for the protective dopamine D4 receptor agonists, but these could prove difficult clinically because of psychoactive side effects. Finally, the general insight of how cells become resistant to oxidative stress will lead to a better understanding of the cause of free radical damage and its role in aging and the various neuropathologies of old age.

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Appended manuscripts (see below)

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The role of Bax in glutamate-induced nerve cell death

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Abstract

The role of the Bax gene product was examined in three forms of cortical nerve cell death in primary cultures. These include spontaneous cell death, oxidative glutamate toxicity, in which exogenous glutamate inhibits cystine uptake resulting in toxic oxidative stress, and ionotropic glutamate receptor-mediated excitotoxicity following a brief exposure to 10 μM glutamate. Primary cortical and hippocampal neuron cultures were established from embryos of Bax –/+ × Bax –/+ matings and the embryos genotyped and assayed for cell death in the three experimental paradigms. Cell death induced by oxidative glutamate toxicity and glutamate-mediated excitotoxicity was not altered in the Bax –/– homozygous knockout animals. In contrast, there was an approximately 50% inhibition of spontaneous cell death. These results suggest that a classical Bax-dependent apoptotic pathway contributes to the spontaneous cell death that takes place when nerve cells are initially exposed to cell culture conditions. A Bax-dependent programed cell death pathway is not, however, utilized in oxidative glutamate toxicity and NMDA receptor-mediated excitotoxicity following a brief exposure to low concentrations of glutamate.

Keywords: Bax, knockout, oxidative glutamate toxicity.
J. Neurochem. (2001) **76**, 295–301.

The Bcl-2 family of proteins has been implicated as a necessary intermediate in the death of a wide variety of cell types caused by a large number of different agents (for a recent review, see Chao and Korsmeyer 1998). Within this family, some members inhibit cell death (such as Bcl-2 and Bcl-x_L), while others promote cell death (for example Bax and Bad). A variety of approaches have suggested that the Bcl-2 family regulates at least some forms of cell death in the nervous system. For example, Bax knockout mice have increased cell numbers within the nervous system relative to control animals, as do animals that overexpress Bcl-2 (Farlie *et al.* 1995; Korsmeyer 1999). In contrast, there is excessive cell death in Bcl-2 knockout mice and a promotion of apoptosis in mice that overexpress Bax (Knudson and Korsmeyer 1997). In nerve cell culture paradigms, there have been a large number of studies that implicate the Bax/Bcl-2 pathway in nerve cell death. The best studied of these is the withdrawal of trophic support from neurotropin-dependent cell cultures (see, for example, Deckwerth *et al.* 1996). Other examples include ionizing radiation-induced apoptosis (Chong *et al.* 2000) and p53-induced apoptosis (Bernard *et al.* 1998; Xiang *et al.* 1998; Cregan *et al.* 1999). In the vast majority of these experimental systems it has been concluded that the Bax pathway is required for the completion of the cell death program.

There are, however, two forms of nerve cell death that have been studied less extensively than trophic factor withdrawal with respect to the potential involvement of the Bax/Bcl-2 pathway. These are a form of oxidative stress-induced cell death caused by glutamate, called oxidative glutamate toxicity (for a review, see Maher and Schubert 2000), and excitotoxicity caused by a brief exposure to low concentrations of glutamate. Ionotropic glutamate receptors have two known roles. They are responsible both for the majority of the excitatory neurotransmission within the CNS (for a review, see Gasic and Hollmann 1992) and also for a great deal of CNS pathology. In cases of stroke or trauma, excessive extracellular glutamate leads to nerve cell death via the activation of NMDA receptors (Rothman and Olney 1986). This phenomenon, which can be reproduced in cell culture (Rothman 1985; Choi 1987), is termed excitotoxicity (Olney 1986). Excitotoxicity is thought to be mediated primarily by the entry of calcium ions through NMDA receptors (Garthwaite and Garthwaite 1986; Choi 1987; Connor *et al.* 1987; Hyrc *et al.* 1997).

Another prevalent form of nerve cell death is that caused by oxidative stress associated with many pathologies (for a review, see Coyle and Puttfarcken 1993). Perhaps the best

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model system for examining oxidative stress in the CNS is oxidative glutamate toxicity. Initially described by Murphy *et al.* (1989), oxidative glutamate toxicity occurs when nerve cells are exposed to high exogenous concentrations of glutamate. Extracellular glutamate inhibits the x_c^- cystine/glutamate antiporter, a protein complex that is responsible for the import of cystine in exchange for the export of glutamate (Sato *et al.* 1999). In the absence of cystine, glutathione (GSH), the major cellular antioxidant, is depleted, leading to oxidative stress and ultimately cell death. Although a number of requirements for cell death via this pathway have been identified, the potential role of the Bax/Bcl-2 family is unknown. In this article we ask if Bax is required for cell death caused by excitotoxicity and oxidative stress, as well as the spontaneous cell death seen following the introduction of nerve cells in culture.

Materials and methods

Cell culture

Primary cultures of cortical neurons that reproducibly die by excitotoxicity were prepared by combining aspects of two published protocols (Rose *et al.* 1993; Dugan *et al.* 1995a,b). E14 Balb/c mouse embryo cortices were minced and treated with 0.1% trypsin for 20 min. Following centrifugation, the cells were resuspended in B27 Neurobasal medium (GIBCO, Boston, MA, USA) plus 10% fetal calf serum and dissociated by repeated pipetting through a 1-mL blue Eppendorf pipette tip. The cells were then plated in 96-well polylysine and laminin-coated microtiter plates in B27 Neurobasal plus 10% fetal calf serum and 20% glial growth-conditioned medium prepared according to Dugan *et al.* (1995a,b). The growth-conditioned medium improved plating efficiency by approximately 30%. Two days later the medium was aspirated and replaced by serum-free B27-Neurobasal medium plus 10 μg/μL cytosine arabinoside. The cultures were used without media change between 7 and 12 days after plating and were essentially free of astrocytes (Brewer *et al.* 1993).

For glutamate toxicity assays, the culture medium was moved with a multichannel pipetter to a new 96-well plate and the cells exposed to 10 μM glutamate in a HEPES-buffered salt solution (HCSS: Rose *et al.* 1993), containing 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose and 20 mM HEPES, pH 7.4. In some cases, 1 μM glycine was included, but this had no net effect on excitotoxic death. After 10 min at 23°C, the HCSS was aspirated and the original growth medium returned to the cells. In all experiments test and control cells were exposed to identical conditioned media.

MTT assay

Cell survival was determined by the MTT [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as described by Liu *et al.* (1997), which correlates with cell death as determined by trypan blue exclusion and a colony-forming assay (Davis and Maher 1994). Twenty hours after the addition of glutamate, 10 μL of the MTT solution (2.5 mg/mL) was added and the cells were incubated for 3 h at 37°C. 100 μL of the solubilization solution (50% dimethylformamide and 20% SDS, pH 4.8) was added to the wells, and the next day the absorption values at 570 nm were measured. The results are expressed relative to the controls specified in each experiment and are expressed as the mean of triplicate determinations plus or minus the standard error of the mean.

Western blotting

For western blotting, cortical cells were collected directly into Laemmli buffer (Laemmli 1970). Cell lysates (30 μg per lane) were resolved in 12% polyacrylamide gels containing SDS and electrophoretically transferred to hybridization membranes (Micron Separations Inc., Westboro, MA, USA). The membrane was first probed with a rabbit anti-Bax antiserum (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 μg/mL and then with horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody at a dilution of 1 : 20 000. The antibody conjugates were detected using a chemiluminescence western blot kit (Amersham, Buckinghamshire, UK).

Bax knockout mice

Heterozygous Bax mice were obtained from Dr Stanley Korsmeyer (Deckwerth *et al.* 1996) and bred both to maintain the colony and as a source of embryos that lack Bax protein. Genomic DNA was extracted from tissue samples (0.5-cm lengths of tail from weanlings, 10–20 mg of brain from embryos) using the DNeasy Tissue Kit (Qiagen Inc., Valencia CA, USA) following the suggested protocol. Relevant segments of DNA were amplified using AmpliTaq DNA Polymerase (Perkin Elmer, Boston, MA, USA) in accordance with the protocol provided by S. Korsmeyer and developed by M. Knudson. The following primers were used: BaxIN5R (Bax Intron 5 reverse primer) 5′-GTT GAC CAG AGT GGC GTA GG-3′; NeoR (Neo/PGK reverse primer) 5′-CCG CTT CCA TTG CTC AGC GG-3′ (because Neo is in a reverse orientation it amplifies in the forward direction with Bax primer IN5R); BaxEX5F (Bax Exon 5 forward primer) 5′-GAG CTG ATC AGA ACC ATC ATG-3′. Reaction conditions were: 5 min at 94°C, cycle: 1 min 94°C, 1 min 62°C, 1.5 min 72°C. The cycle was repeated 30 times followed by a 7-min elongation step at 72°C. Reactions were in 25-μL volumes using all three primers. PCR products were resolved with 1.5% agorose gels.

Results

While the role of Bax has been studied extensively in several experimental paradigms for apoptosis, there has been no work on its role in nerve cell death caused by oxidative glutamate toxicity and only one study that examined the role of Bax and P53 in nerve cell death caused by continuous exposure to relatively high concentrations of glutamate/kainate (Xiang *et al.* 1998). There is, however, little published material examining the role of Bax in excitotoxicity. We therefore examined the potential role of Bax in nerve cell death by asking how cells isolated from the nervous system of Bax knockout mice respond to oxidative stress, NMDA receptor-mediated excitotoxicity

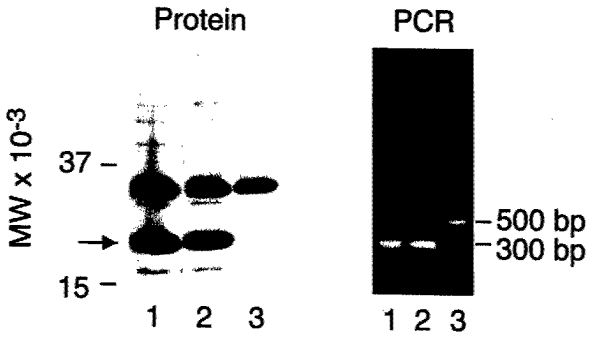

Fig. 1 Bax is not expressed in homozygous Bax-deleted animals. The genotype of individual embryos was determined as described in Materials and methods using PCR primers. Cortical tissue from the same animals was separated on SDS acrylamide gels and immunoblotted with anti-Bax antiserum. Lane 1, Bax +/+ Lane 2, Bax –/+ Lane 3, Bax –/–. Arrow indicates position of Bax in the Western blots. The bands above 25 000 MW are non-specific. PCR detection of genotype. Lane 1, Bax –/+ Lane 2, Bax +/+ Lane 3, Bax –/–.

and spontaneous cell death in culture. If Bax is involved in the cell death mechanism, then it would be expected that cells lacking this protein would die less efficiently than wild-type cells. As controls we used staurosporine-induced apoptosis, processes known to be regulated by the Bax/Bcl-2 pathways (Rodriguez *et al.* 1996). The following paragraphs present the results of experiments designed to test the role of Bax in nerve cell death.

Bax expression is not required for excitotoxic or oxidative stress-induced nerve cell death

To determine if Bax expression is required for the forms of cell death that occur in NMDA receptor-mediated excitotoxicity as well as in oxidative glutamate toxicity, a model for oxidative stress (Murphy *et al.* 1989; Tan *et al.* 1998a,b), E14 cortical neurons were cultured either for 2 days (oxidative glutamate toxicity) or 10 days (excitotoxicity) and assayed for sensitivity to glutamate. Cells from individual embryos were used and their genotype determined by PCR analysis. Figure 1 shows PCR patterns and Bax protein expression in cortical cells from a few embryos, confirming the absence of Bax protein expression in the homozygous Bax-deleted animals. Bax is present in the heterozygous animals as expected.

The excitotoxic assay, based upon the activation of NMDA ionotropic glutamate receptors in 10-day-old cortical cultures (Schubert and Piasecki, submitted to *J. Neurosci.*) showed that in wild-type, heterozygous and homozygous Bax knockout cells there is no difference in cell death caused by low concentrations of glutamate (Figs 2 and 3). The excitotoxic response is completely blocked by the NMDA receptor antagonist AP-5, showing that the cell death cascade is at least initiated by NMDA receptor activation.

In contrast to older cultures, mouse cortical neurons cultured for only 2 days express very low levels of any class of ionotropic glutamate receptors and therefore do not respond to excitotoxic insults (Murphy *et al.* 1989; Schubert and Piasecki, submitted to *J. Neurosci.*). Although these cells lack ionotropic receptors, higher concentrations of glutamate block cystine uptake, causing a depletion of glutathione and a form of programed cell death (Murphy *et al.* 1989; Tan *et al.* 1998a,b). When cultures from Bax –/– or Bax +/+ embryos are continuously exposed to concentrations of glutamate between 0.1 and 5 mM for 24 h there is half-maximal cell death at 1 mM glutamate in both sets of cultures. It can therefore be concluded that the expression of Bax is not required for nerve cell death observed in oxidative stress or NMDA receptor-mediated excitotoxicity.

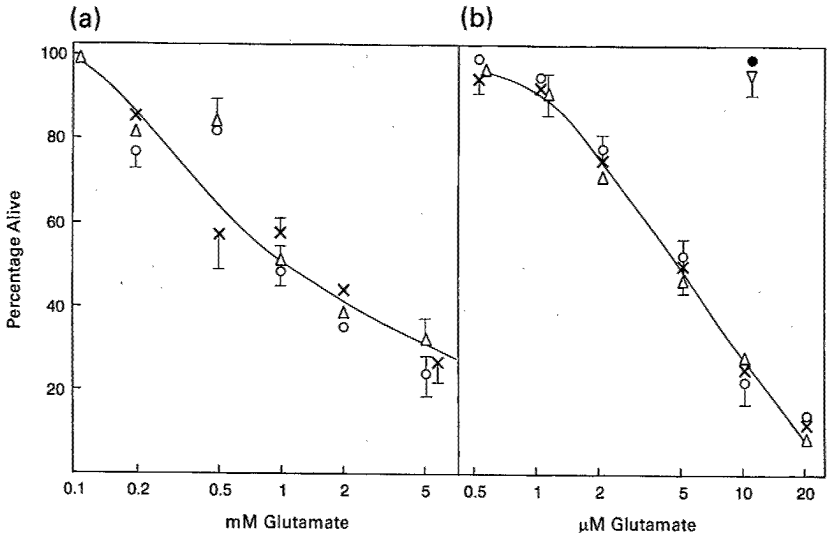

Fig. 2 Oxidative glutamate toxicity and excitotoxicity. Cells from individual E14-day embryos of Bax heterozygous matings were plated in 96-well microtiter dishes and assayed for either (a) oxidative glutamate toxicity in 2-day cultures lacking ionotropic glutamate receptors or (b) excitotoxicity in 10-day-old cultures expressing ionotropic glutamate receptors as described in Materials and methods. Pieces of brain tissue from each embryo were used to isolate DNA and to determine the Bax genotype of the individual animals. Results are presented as the percentage of the control (non-treated) cell viability at each concentration of glutamate. ○, Bax –/–; ×, Bax +/+ △, Bax –/+. ●, 100 μM AP-5 Bax –/–; ▽, 100 μM AP-5 Bax +/+. The results are presented as the mean ± SEM ; $n = 6$.

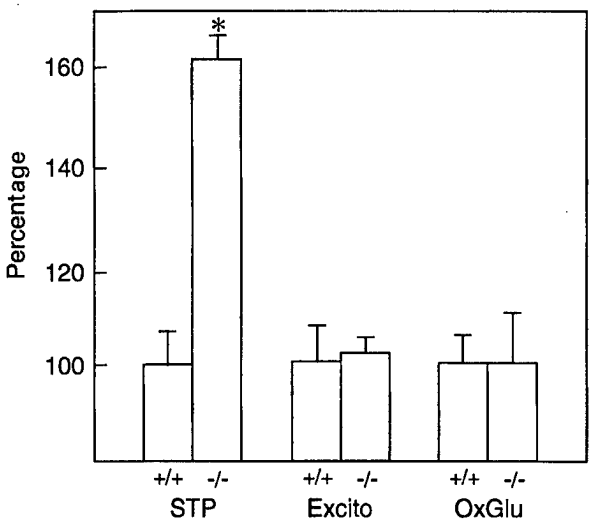

Fig. 3 Cell death in Bax knockout mice. Cortical nerve cells from individual E14 embryos resulting from a Bax –/+ × Bax –/+ mating were assayed for excitotoxicity, oxidative glutamate toxicity or staurosporine-induced cell death as described in Materials and Methods. The results are presented as the mean ± SEM for at least 5 embryos. *Statistically different from control ($p < 0.01$). The data are calculated as the percentage change in cell viability relative to the control cultures derived from wild-type mice (Bax +/+) with the following conditions: staurosporine (STP, 200 nM); excitotoxicity (Excito, 5 μM glutamate); oxidative glutamate toxicity (OxGlu, 2 mM glutamate).

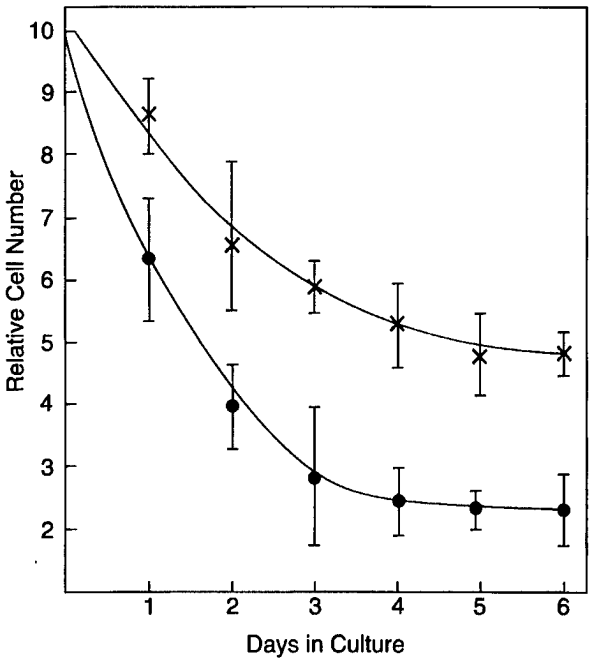

Fig. 4 Time course of cell loss in cortical primary cultures. E14 cortical neurons were plated on polylysine at 5×10^5 cells per 35-mm tissue culture dish and viable cells determined daily as described in Materials and methods. The relative cell number is plotted against days in culture. ×, Bax –/–; ●, Bax +/+.

Bax expression is required for staurosporine induced death

As a positive control to establish that the cortical neurons were in fact functioning properly, an additional cell death pathway was examined in which a role for Bax was established. Staurosporine and 2-day-old cultures of cortical neurons were used. Staurosporine, a potent phosphatase inhibitor, causes Bax-dependent apoptosis (Ackermann *et al.* 1999). Figure 3 shows that the genomic deletion of Bax promoted cell survival from staurosporine-induced cell death. Therefore it is clear that within the same population of cells both Bax-dependent and Bax-independent forms of programed cell death can occur.

Bax expression is required for some of the initial spontaneous cell death in cortical neuron cultures

With the culture conditions frequently used for primary cultures of CNS tissue, there is usually a rapid die-off of neurons during the first 2 days in culture, resulting in the loss of over 50% of the cells (Yankner *et al.* 1990). If this process is caused by a classic apoptotic mechanism, possibly growth factor withdrawal, then it would be expected that this loss would be significantly reduced in cells from homozygous Bax-deleted mice relative to their wild-type littermates. Figure 4 shows that there are approximately twice the number of surviving cortical neurons in culture after 4 days in cultures from Bax –/–knockout mice than in the Bax +/+ controls.

Discussion

The following conclusions may be drawn from the above data.

1 The deletion of the Bax gene partially rescues cortical neurons from the spontaneous cell death that occurs once dissociated cells are placed in cell culture.

2 Bax gene expression is not required for the death of cortical neurons caused by NMDA glutamate receptor-mediated excitotoxicity or glutamate-induced oxidative stress (oxidative glutamate toxicity).

3 The deletion of Bax does, however, protect cortical neurons from staurosporine-induced cell death, suggesting that Bax-dependent apoptosis is responsible for the majority of the cell death in this system.

When CNS neurons are dissociated and placed in cell culture, there is a rapid die-off of cells until the cultures reach a stable cell number (see, for example, Yankner *et al.* 1990). The cell depletion is probably due to the loss of neurotropic support, suggesting that death occurs by an

apoptotic mechanism analogous to that caused by the withdrawal of NGF from cultured sympathetic neurons. Since Bax is required for cell death to occur in NGF-deprived sympathetic neurons (Deckwerth *et al.* 1998) as well as hyperpolarized cerebellar granule cells (Miller *et al.* 1997), it is likely that at least some of the spontaneous cell death in cortical primary cultures should be rescued by the deletion of Bax. Figure 4 shows that there is indeed a 2.5-fold increase in cell number after 4 days in culture for Bax –/– cortical neurons relative to wild-type Bax +/+ cultures.

The activation of the NMDA class of glutamate receptors causes rapid cell death by a process that has primarily been defined as necrosis, although some data have suggested that apoptotic mechanisms may also be employed (Ankacrona *et al.* 1995). The influx of Ca^{++} through open NMDA channels is thought to initiate the excitotoxic cascade, but the overproduction of reactive oxygen species (ROS) and mitochondrial dysfunction are also involved (Dugan *et al.* 1995a,b; Patel *et al.* 1996; Schinder *et al.* 1996). Figures 2 and 3 show that unlike spontaneous cell death in the primary cultures, excitotoxic cell death initiated by a brief 10-min exposure to 10 μM glutamate is not altered by the deletion of the Bax gene. Cell death in this system is exclusively mediated by NMDA receptors and is completely blocked by the NMDA-specific antagonist AP-5 (Fig. 2b: Schubert and Piasecki, submitted to *J. Neurosci.*). It is therefore unlikely that nerve cell death via excitotoxicity involves the classical Bax-dependent apoptotic pathway. These data are in agreement with those of Miller *et al.* (1997), who showed that the death of cerebellar granule cells initiated by NMDA (a form of excitotoxicity) is also unaffected in cells from Bax –/– mice relative to control Bax +/+ mice.

The above data are, however, at odds with those of another experimental paradigm that concludes that Bax expression is required for excitotoxic cell death (Xiang *et al.* 1998). In the Xiang manuscript, cortical neurons were exposed to high (50 μM) concentrations of glutamate or kainate continuously for 3 days before cell viability was determined. In contrast, our data were obtained after a brief 10-min exposure to a low 10 μM glutamate, and cell death monitored 24 h later. It has been well established that both the mechanism and the type of cell death that occurs both *in vivo* and in cell culture depends upon both the concentration and the duration of exposure to glutamate or glutamate agonist (for a review, see Choi 1992). Rapidly triggered excitotoxicity, as used in this study, and slowly triggered excitotoxicity, as described by Xiang and colleagues, are probably mediated by different sets of ionotropic glutamate receptors and result in physiologically distinct cell death pathways (Choi 1992). It is therefore not unlikely that the Bax dependencies for cell death in the two experimental systems are different.

Oxidative glutamate toxicity is a form of glutamate-induced cell death that is independent of ionotropic glutamate receptors, but shares some intermediate steps with excitotoxicity such as ROS production (Murphy *et al.* 1989). Most mammalian cells synthesize little or no cysteine, and are therefore dependent upon the import of extracellular cystine for both amino acids. The inhibition of the unique cystine/glutamate antiporter (Sato *et al.* 1999) by high concentrations of exogenous glutamate reduces intracellular cysteine and leads to severe oxidative stress and cell death. This pathway can, in fact, be a component of the excitotoxicity cascade when there is a high concentration of extracellular glutamate (Schubert and Piasecki, submitted to *J. Neurosci.*). Oxidative glutamate toxicity has several features of classical apoptosis, including the requirement for macromolecular synthesis and for caspase activation (Tan *et al.* 1998a). However, the morphological criteria for classical apoptosis are not seen, nor is there DNA breakdown (Tan *et al.* 1998a, 1998b). Despite the overlap of the oxidative glutamate toxicity pathway with some aspects of classical apoptosis, there is no alteration in the kinetics or glutamate concentration dependence of cell death in cortical nerve cells derived from Bax –/– or Bax –/+ mice relative to Bax +/+ animals (Figs 2 and 3). It is therefore unlikely that Bax is involved in cell death caused by oxidative glutamate toxicity. It has, however, been shown that the overexpression of Bcl-2 can protect PC12 cells from oxidative glutamate toxicity (Behl *et al.* 1993). This is not surprising, since Bax and Bcl-2 can function independently to regulate cell death (Knudson and Korsmeyer 1997). Bcl-2 can act as a pro-oxidant and can protect cells from oxidative stress when overexpressed by upregulating antioxidant defense mechanisms, therefore making cells more resistant to additional insults (Steinman 1995).

In summary, the above data derived from primary cortical cultures of Bax –/– mice show that the Bax gene product is not required for nerve cell death pathways initiated by oxidative glutamate toxicity or by excitotoxicity initiated via the short-term activation of NMDA receptors by low concentrations of glutamate. They are in agreement with the exceptionally heterogeneous literature on cell death within the nervous system which shows that nerve cells can die by a variety of cell death pathways. The initiation of each pathway is, however, strictly dependent upon the nature of the insult.

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CMLS Cellular and Molecular Life Sciences

Review

Signaling by reactive oxygen species in the nervous system

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Abstract. Free radicals and reactive oxygen species (ROS) are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis. Cells contain multiple sites for ROS production and a few mechanisms for their degradation. Which of these sites is activated by a given stimulus may play a role in dictating the subsequent downstream effects of the ROS generated on cellular function. Even when the ultimate outcome is similar, such as when ROS production leads to cell death, the specific cellular changes can be quite different depending on the initial stimulus and the type of cell involved. These data, along with the evidence that ROS can modify a number of intracellular signaling pathways including protein phosphatases, protein kinases and transcription factors, suggest that the majority of the effects of ROS on cells are through their actions on signaling pathways rather than via nonspecific damage of intracellular macromolecules.

Key words. ROS; apoptosis; necrosis; glutathione; H_2O_2; transcription factors; tyrosine phosphatases; protein kinases.

Introduction

Although the potential toxicity of molecular oxygen (O_2) was recognized in the 18th century by Lavoisier, Priestley and Scheele, it was not until 1954 that Rebeca Gerschman did the compelling experiments to show that O_2 toxicity was mediated by oxidizing free radicals [1]. Nineteen years later Babior demonstrated that the superoxide radical ($O_2^{\cdot -}$) is produced by leukocytes as a defense mechanism against bacteria [2], and the scientific community began to appreciate the possibility that free radicals and reactive oxygen species (ROS) may play significant roles in cell physiology. Today the experimental literature is replete with data showing that this group of highly reactive molecules is involved in everything from apoptosis and necrosis to carcinogenicity and aging. Clearly, a single review can cover but a small fraction of this enormous and rapidly expanding area of scientific endeavor. Since the authors' expertise is in the areas of neurobiology and growth factors, this review will focus upon the role of ROS in stress responses and cell death within the central nervous system (CNS). We will not discuss nitric oxide nor will we discuss in detail the enormous literature on p53 and nuclear factor κB (NF-κB) in apoptosis (see for example [3, 4]). Initially, a primer on free-radical chemistry will be presented, followed by a discussion of potential sources of free radicals and ROS in mammalian cells. This will be followed by an outline of our current mechanistic knowledge of the physiological roles of ROS and free radicals in cell death and stress. An

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attempt is made to draw attention to pathways that probably play a central role in most forms of cell death, but have not received the attention they deserve. Finally, we will use the term programmed cell death in reference to all forms of death which require active cellular participation (i.e. gene transcription and/or enzyme activation) in order for the cells to die in response to a stressor. A justification for this use of the term will be presented after the discussion on apoptosis and necrosis.

Reactive oxygen species and free radicals

Oxidation is the loss of electrons by an atom or molecule, and therefore an oxidizing agent is good at absorbing electrons from the molecule or atom it oxidizes. Free radicals, which contain one or more unpaired electrons, and ROS, which are radical derivatives of molecular oxygen as well as hydrogen peroxide (H_2O_2), are therefore good oxidizing agents. Biologically relevant ROS include the superoxide radical ($O_2^{\bullet -}$), hydroperoxyl radicals ($HO_2^{\bullet}$), H_2O_2 and the hydroxyl radical (·OH). Of these, the hydroxyl radical is the most damaging, for it reacts with many macromolecules with a rate constant approaching diffusion-controlled. In contrast, $O_2^{\bullet -}$ reacts very slowly, and some consider it to be a sink for radical electrons. Additional members of this family are derived from the interaction of carbon- and nitrogen-based radicals with O_2. These include organic peroxides, alkoxyl (RO·) and peroxyl (RO_2·) radicals, and peroxynitrite ($ONOO^-$) and nitric oxide (·NO). The extensive reactions which occur between this family of molecules and the components of cells has been thoroughly reviewed [5, 6] and will not be discussed here except to emphasize a point of discussion. All free radicals and many ROS derivatives can, however, cause extensive damage to macromolecules through the formation of adducts, the destruction of unsaturated C–C bonds, and the oxidation of disulfides. These unique properties have allowed ROS to evolve into functional roles in cell death and as exquisitely sensitive signaling molecules within cells.

What are the sources of ROS?

There are two ubiquitous sources of ROS in eukaryotic cells. The first source of free radicals is from the effect of ionizing radiation on water, which generates a variety of initial products, many of which can go on to form additional reactive molecules.

$$H_2O \rightarrow \rightarrow \rightarrow e_{aq}^-,\ \cdot OH,\ \cdot H,\ H_3O^+,\ H_2,\ H_2O_2$$

Since both cosmic and terrestrial background radiation contribute to this source of ROS, it is clear why organisms have evolved elaborate defense systems to deal with the problem of ROS production (to be discussed later). The second source results from the development of aerobic metabolism about 1.5 million years ago. Figure 1 is a schematic representation of the flow of electrons through mitochondria, a process that is known in great detail (for review see [7]). While the goal of the respiratory chain is to use metabolites to produce energy from the reduction O_2 to water, this series of reactions is inefficient, resulting in the production of ROS from the interaction of molecular oxygen with free electrons. It was initially shown that isolated mitochon-

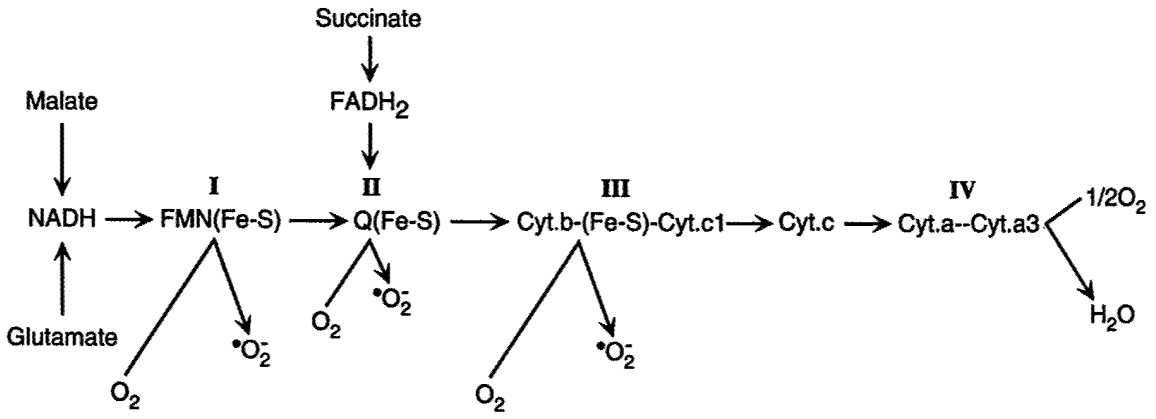

Figure 1. Production of superoxide by the mitochondrial electron transport chain. Although it is generally agreed that mitochondria produce significant amounts of superoxide under physiological conditions, the relative contribution of each complex is controversial. However, the consensus is that the majority of the superoxide comes from complexes I and III, as indicated in the figure.

dria produce H_2O_2 (derived from $O_2^{\cdot -}$) from both nicotinamide adenine dinucleotide (NAD)- and flavin adenine dinucleotide (FAD)-linked substrates [8]. Although still controversial, it is currently believed that the major sources of mitochondrial H_2O_2 production are from the ubiquinone sites in complexes I [NADH (the reduced form of NAD)-ubiquinone reductase] and III (ubiquinal-cytochrome C reductase), with a less substantial contribution from complex II [8–11]. There is no evidence that ROS are generated during the final step of O_2 reduction to H_2O via cytochrome oxidase. The 'leak' of electrons from the upstream components of the chain to O_2 results in the formation of $O_2^{\cdot -}$. $O_2^{\cdot -}$ is then dismutated to H_2O_2 by mitochondrial superoxide dismutase (SOD), and H_2O_2 is converted in the presence of iron to the highly reactive hydroxyl radical, a reaction described by Fenton in 1894.

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {\cdot}OH$$

When electron transfer through mitochondria is reversed by the presence of excess ATP and succinate, there is a dramatic increase in $O_2^{\cdot -}$ production [11], suggesting that a high ADP/ATP ratio due to energy utilization may minimize ROS production by mitochondria. Such a condition may contribute to the observation that calorie restriction promotes longevity in some animals.

Although the physiological source of ROS in most cell death pathways appears to be mitochondria, many other sources of ROS have been identified in the CNS. Indeed, several are more potent than mitochondria. The best studied of these is the NADPH (reduced form of NAD phosphate) oxidase of macrophages and CNS microglia (for review see [12]). Although the exquisitely regulated NADPH oxidase complex was initially thought to be confined exclusively to macrophages and microglia, NADPH oxidases have been implicated in growth factor signaling in a variety of cell types (see for example [13–15]), including nerve cells [16]. The latter manuscript showed that the five required subunits of NADPH oxidase are present in sympathetic neurons and that the activity of this complex is required for the programmed cell death associated with NGF withdrawal. These data therefore expand the potential number of sources for ROS signaling and perhaps toxicity within the nervous system. Other, less cell type specific, sources of ROS include the large numbers of oxidases associated with peroxisomes, endoplasmic reticulum and cytoplasm. These include lipoxygenases and cyclooxygenases, cytochrome P450 oxidases, monoamine oxidases, and nitric oxide synthase. In addition, there are nonenzymatic sources such as catecholamine autoxidation. These abundant sources of ROS, as well as the brain's extraordinarily high rate of metabolism due to the requirement for pumping ions associated with synaptic communication (the brain uses about 20% of the body's total O_2 intake despite the fact that it has only about 2% of the total body weight. Therefore the respiration rate is 10 times higher than that of average tissue) has forced CNS cells to evolve an elaborate machinery for protection from ROS. In addition, the high levels of the transition metals iron and copper in certain neuronal cells support Fenton chemistry to generate ·OH, and the many unsaturated lipids in nerve cell membranes are susceptible to lipid peroxidation.

Defense systems

Due to both the background generation of ROS from ambient radiation and the endogenous generation of cellular ROS outlined above, cells have evolved an elaborate series of mechanisms to minimize ROS presence and reduce damage if it occurs. Figure 2 outlines the pathways involved in ROS generation and control. Within the CNS, it is likely that the major enzymatic system for maintaining low levels of the more dangerous ROS is glutathione peroxidase (GPx) and the enzymes associated with maintaining high levels of intracellular reduced glutathione (GSH). GSH is a tripeptide (γ-glu-cys-gly) characterized by De Rey-Pailhade over 100 years ago [17]. Since the glutathione thiol has a low oxidation potential and therefore reducing power, it is a potent antioxidant. Its intracellular concentration is between 2 and 10 mM, of which 99% is normally in the reduced state [99% GSH vs. 1% glutathione disulfide (GSSG)]. Both GPx and catalase can reduce peroxides (H_2O_2 or ROOH) to water or organic alcohols (ROH), but in the nervous system the major player seems to be GPx, for it is both more abundant, particularly in mitochondria, and has a higher turnover rate than catalase. Superoxide dismutase (SOD) is a double-edged sword, for it converts the relatively innocuous $O_2^{\cdot -}$ to H_2O_2, which is readily degraded to the very reactive ·OH via the Fenton reaction. Since Fenton chemistry requires multivalent iron or copper to mediate electron transfer, sequestration of these metals by molecules such as ferritin (which retains about 4500 iron ions per molecule of protein) can also serve as a defense mechanism against free radical damage and therefore should be considered an antioxidant. The antioxidant vitamins E and C are also important in the CNS [18]. Other enzymes such as heme oxygenase and quinone reductase can mitigate radical formation, and a number of cysteine-rich proteins such as metallothionein, thioredoxin (Trx) and redox factor 1 can behave like GSH to maintain the redox status of cells and minimize oxidative stress.

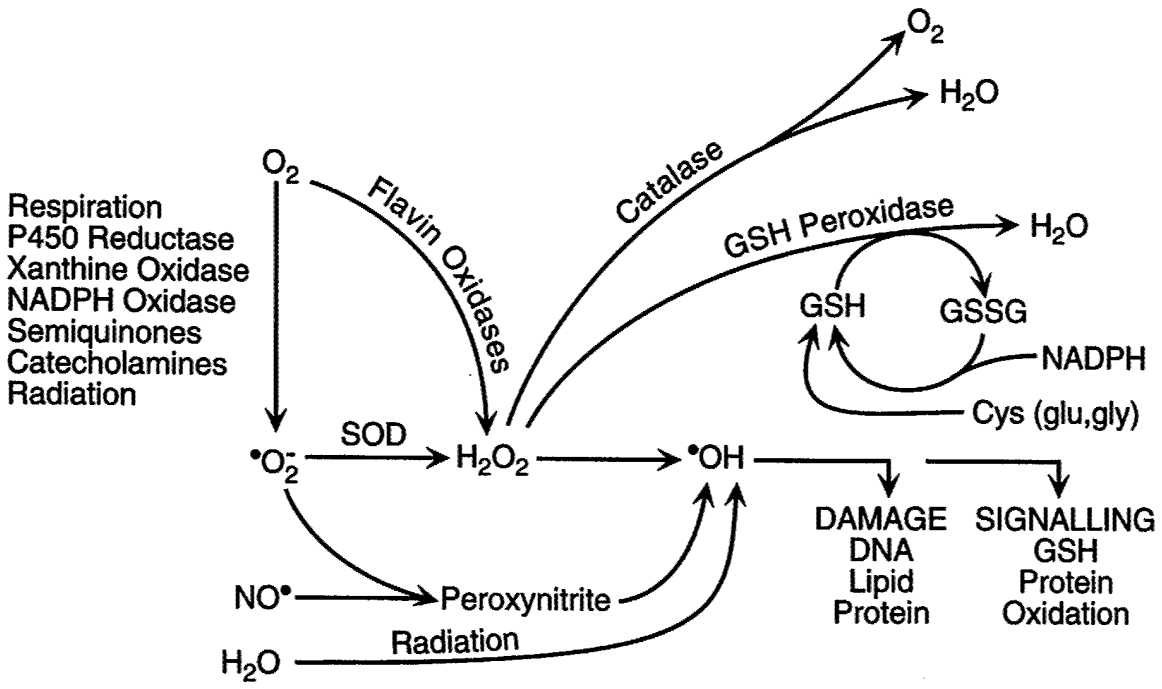

Figure 2. Sources of ROS and mechanisms for their removal from cells. SOD, superoxide dismutase; GSH, glutathione.

What are 'oxidative stress' and 'redox status'?

It has generally been assumed that oxidative stress and an imbalanced redox homeostasis are functionally the same with respect to generation and consequences—they occur when there is either an overproduction of ROS or a deficiency in the sum total of the antioxidant systems. This may not, however, be the case, for it is likely that under some forms of stress there is a deficiency in total reducing potential (e.g. low GSH, low Trx) in the absence of significant ROS production, and vice versa. Although ROS can lead to the oxidation of protein thiols, it is also likely that thiol-based redox molecules such as GSH and Trx can act directly as signaling molecules in the absence of excessive ROS. For example, when there is an excessive accumulation of GSSG, it can inactivate a number of enzymes and other proteins (e.g. the transcription factor AP-1, see below) by forming mixed disulfides.

$$\text{active } E-SH + GSSG \rightarrow \text{inactive } E-S-SG + GSH$$

Conversely, ROS and other radicals may serve as 'second-message' signals independently of the overall status of thiol redox state, albeit GSH may be lowered by the necessity of detoxifying ROS. Other unique ROS signaling pathways may involve tyrosine nitration [6]. Even within the ROS family, different sensing molecules have been identified in bacteria for H_2O_2 (OxyR) and $O_2^{\bullet-}$ or NO (SoxR) [19]. It can therefore not be assumed that delivering prooxidant stress by different ways either experimentally (e.g. organic vs. inorganic peroxides) or pathologically (ischemia vs. Parkinson disease) will produce the same set of physiological responses. Indeed, the major function of ROS and redox status in the cell is likely to be signaling rather than overt killing.

Bcl-2

Bcl-2 is one of the first proteins shown to regulate apoptosis. It is localized to the outer membranes of mitochondria and nuclei, and forms heterodimers with a family of proteins which contain a unique helical domain called BH3. Bcl-2 and its binding partners may alter ROS production and are clearly involved in some examples of programmed cell death. It is, in fact, frequently assumed that if cell death is inhibited by the overexpression of Bcl-2, then the cells die by a classical apoptotic mechanism. However, since the overexpression of Bcl-2 in cell lines or transgenic animals is often 10-fold or more higher than would ever be found in a normal cell, is the extrapolation from Bcl-2 inhibition to

a specific cell death mechanism valid? New data on the mechanism of Bcl-2 protection make it unlikely that the inhibition of cell death by Bcl-2 is sufficient to warrant the conclusion that death is via classical apoptosis. By expressing Bcl-2 in Bcl-2-negative lymphoma cells, it was shown that Bcl-2 causes about a twofold increase in basal mitochondrial H_2O_2 production [20]. In fact, when Bcl-2 is expressed in bacteria, it acts as a prooxidant [21]. When apoptosis is induced in the non-Bcl-2-expressing cells by C6-ceramide or by tumor necrosis factor (TNF-α), there is an increase in H_2O_2 accumulation. This increase does not occur in the Bcl-2-expressing cells. The mechanism whereby Bcl-2 increases H_2O_2 is probably by increasing the activity of SOD [21, 22]. The selection of stress-resistant cells by growth in the presence of agents which cause cells to produce ROS, such as amyloid or glutamate, also selects for populations with elevated catalase, GPx and other antioxidant activities [23, 24]. Direct selection by growth in the presence of peroxides produces a similar phenotype. Thus, if there is a positive (linear) relationship between the amount of Bcl-2 expression and the induction of ROS defenses, then any process involving a ROS intermediate, be it necrosis or apoptosis, would be blocked. It is likely that Bcl-2 overexpression increases endogenous oxidative stress defense mechanisms which then allow the cells to degrade the ROS produced by TNF-α or ceramide, preventing ROS accumulation. Therefore, the elevated expression of Bcl-2 will produce a more ROS-resistant cell, and one resistant to many forms of oxidative stress [25].

Apoptosis or necrosis?

The initial and defining distinction between necrosis and apoptosis was made on the basis of the ultrastructural characteristics of the dying cell and the observation that cells with an apoptotic ultrastructure tended to be phagocytosed. Apoptosis was defined as 'a mechanism of controlled cell deletion' and as 'an active, inherently programmed phenomenon' [26]. It was identified in tissues by cell shrinkage, fragmentation and chromatin condensation: dying cells are phagocytosed and there is little inflammation. In contrast necrosis, a term for cell death which has been around for over a century, is thought to be an 'irreversible disturbance of cellular homeostatic mechanisms' and is morphologically recognized by cell swelling and mitochondrial destruction. In 1980 Wyllie et al. [27] put together the ultrastructural data of Kerr with the observations from several laboratories that the irradiation of lymphocytes caused the 'laddering' of genomic DNA, into a coherent package of biochemical and morphological criteria for apoptosis. This paper initiated the current wave of interest in the subject. Once apoptosis was studied in additional cell culture systems, a number of other criteria, such as the requirement for de novo protein synthesis, entered the literature and the data on caspases were transferred from *Caenorhabditis elegans* to mammalian cells.

Although it was initially assumed that apoptosis and necrosis were unrelated events, over the last few years exceptions have been found for essentially all of the criteria which initially defined apoptosis, and it now appears to us that there is, in fact, no fundamental distinction between the two processes. Everyone would agree that for apoptosis to occur the cell must somehow participate actively in its own demise (this was indeed part of Kerr's definition), yet this was erroneously assumed not to be the case with necrosis. For example, the depletion of intracellular GSH by buthionine sulfoximine (BSO), a GSH-synthetase inhibitor, causes cell death which appears by all earlier criteria to be necrotic [28]. More recently, however, it has been shown that nerve cell death initiated by GSH depletion is, in fact, a form of programmed cell death which requires macromolecular synthesis, caspase activation, ROS production, and Ca^{++} mobilization [29, 30]. There is, however, no DNA laddering, and the morphology of the dying cells appears more similar to what was initially defined as necrosis [28, 30]. Since both apoptosis and necrosis require a ROS intermediate, it seems to be a mistake to make a global distinction between necrosis and apoptosis at all—they are the ends of a continuum. Indeed, even the initial morphological criteria used to distinguish apoptosis from necrosis are somewhat dubious, and these clearly cannot be applied to cell culture work. For example, the lack of inflammation in apoptosis relative to necrosis may simply reflect the number of dying cells, for apoptotic death usually occurs sparingly within populations. In contrast, classic necrotic cell death is frequently associated with pathological events and usually occurs en masse, resulting in a much greater release of cytokines and other proinflammatory molecules. In fact, when there is extensive localized apoptosis, such as occurs in the leg bud of the chick embryo, there is a very large inflammatory response (for an innovative review of this and other issues being discussed here see [31]). The other defining aspect of apoptosis is cell fragmentation associated with cell shrinkage. However, this combination of phenomena is only seen in animal tissues in vivo, and the cell fragments (apoptotic bodies) are only seen inside other cells. Therefore, the tendency of cells toward shrinking (apoptosis) or swelling (necrosis) could simply reflect the inherent ability of the surrounding cells to phagocytose the cytoplasmic protrusions which occur on the surface of all dying cells. In fact, some cells do swell during apoptosis [31]. It seems that the driving force to

categorize behaviors into extremes is based upon two socioeconomic issues rather than upon science. First, apoptosis is clearly a much flashier way to die than what has classically been termed necrosis, and papers on apoptosis are more likely to be published in high-profile journals. Second, companies promote kits which claim to identify apoptotic cells, even though in actuality these kits do not make the distinction between any classes of dying cells. For example, TUNEL labeling of DNA identifies cells with all kinds of fragmented DNA, but also viable cells undergoing extensive DNA repair or in the process of division. Conversely, BUdR, which is though to label dividing cells, can also label dying cells partaking in DNA repair. Therefore, unless a cell is killed outright by an environmental event or critical aspects of its metabolism are directly inhibited, it cannot be assumed that any type of cell death occurs by a passive necrotic mechanism.

Finally, a few sentences about what is meant by the term programmed cell death. This term was initially used in the context of subpopulations of cells dying at a certain time point during embryonic development. For example, during the development of the mammalian nervous system, large populations of nerve cells die by classical apoptosis at the exact same time and place in each individual. Because of the reproducibility of this phenomenon, it was assumed that the cell death was genetically programmed and not externally driven. We now know, however, that this type of cell death in the nervous system is determined by competition for target tissues and trophic factors and not by any inherent death program within the cells. Therefore, the term programmed cell death as it has been used historically is mechanistically incorrect in many, if not most, cell deaths which occur during development. It seems to us that the term should now be used in the broader sense of any cell death mechanism which requires active cellular participation in terms of macromolecular synthesis or enzyme activation.

Excitotoxicity

Although glutamate is the most abundant neurotransmitter, its extracellular concentration must be maintained below 1 μM, for it is toxic above this level (see for example [32]). The maintenance of low extracellular glutamate is accomplished by five isoforms of sodium-dependent glutamate transporters expressed on neurons and astrocytes (for review see [33]). Glutamate uptake by astrocytes is inhibited by concentrations of H_2O_2 which are probably similar to those generated by CNS pathologies such as stroke, ischemia and trauma. A common target of ROS is the oxidation of sulfhydryl groups, and it is thought that this is the mechanism which leads to the inactivation of the glutamate transporters under conditions of oxidative stress [33].

As with apoptosis, cell death initiated by the activation of ionotropic glutamate receptors, termed excitotoxicity [34], seems to be mediated by mitochondria and ROS. The precise mechanisms of cell death are, however, unclear (see for example [35]). Ca^{++} influx through activated *N*-methyl-D-aspartate (NMDA) receptors (or D,L-amino-3-hydroxy-5-methyl-4-isoazolepropionate [AMPA]/kainate receptors [36]) initiates the cell death pathway, for both NMDA receptor activation and extracellular Ca^{++} are required for the rapid increase in intracellular Ca^{++} [37, 38]. Excess intracellular Ca^{++} uncouples electron transfer from ATP synthesis, and results in the generation of ROS, a process which is autocatalytically augmented by ROS [39]. Ca^{++} also causes the mitochondria to become depolarized, a process which is inhibited by the application of cyclosporin A, which plugs the mitochondrial transition pore [40]. While these results show that the mitochondria and presumably mitochondrially generated ROS are required for excitotoxic cell death, they do not establish a formal role for either in the cell death pathway. It is not known, for example, whether the ROS and/or Ca^{++} are sufficient to lyse the cells, or if additional steps are required before the cells die. Finally, NMDA, but not kainate and AMPA receptors, are also directly sensitive to redox changes, but in the opposite manner of glutamate transporters. Oxidation results in a decrease in Ca^{++} flux through activated NMDA receptors, which may be an evolutionarily derived protective mechanism since Ca^{++} flux through NMDA channels is thought to initiate the excitotoxicity cascade.

Oxidative glutamate toxicity: a necrotic form of apoptosis?

Cell death has been studied in dozens of clonal cell lines, primary cultures and animals using an equally large number of reagents and conditions to initiate death. The outcomes of these experiments are usually catalogued as either apoptosis or necrosis based upon a very limited number of assays. Given that there are exceptions to all criteria for apoptosis, we have argued above that it is a mistake to pigeonhole the death of a cell into one category or another. It is, however, important to understand the underlying mechanisms which lead to cell death. Similarly, most reviews lead the casual reader to believe that apoptosis takes place by the same series events, involving the same cast of molecules, in all cell types. This conclusion is certainly not valid, and in fact, even the same triggering reagent can lead to a different series of events depending upon the concentration and exposure time. To emphasize the

points that death which looks morphologically like necrosis may not be, and that the cast of molecules involved in death can be quite different from the more classical Bcl2/Bax paradigm, we will devote a few paragraphs to a unique form of nerve death in primary CNS neurons and the HT22 hippocampal cell line which could be classified as either necrosis or apoptosis, depending upon the assays employed in its definition. However, it is clearly a form of programmed cell death when studied in detail.

HT22 cells were derived from the mouse hippocampus by SV40 transformation and are probably phenotypically most similar to neuronal precursor cells. They lack ionotropic glutamate receptors, but are killed by exogenous glutamic acid in the millimolar range. The initiating event in this form of glutamate-mediated cell death is the blockade of cystine uptake into the cell via the inhibition of the glutamate/cystine antiporter by exogenous glutamate [41]. This antiporter, which has recently been cloned [42], normally transports the oxidized form of the essential amino acid cysteine down its concentration gradient into cells, coupled with the export of intracellular glutamate. High extracellular glutamate blocks this process, depriving the cell of cystine. Inside the cell, cystine is reduced to cysteine, which is a substrate for glutathione synthesis. In the absence of GSH, cells become oxidatively 'stressed', produce large amounts of ROS, and die—a process termed oxidative glutamate toxicity [43]. The result of the insult could be classified as either necrosis or apoptosis, depending upon the assay. If this type of cell death occurred during CNS development or following CNS trauma and was assayed exclusively by ultrastructural methods, it would certainly be classified as necrosis by the standard morphological criteria (see for example [28]). By biochemical criteria, however, it closely resembles apoptosis, although it lacks the involvement of the Bcl2/Bax pathway (unpublished).

When primary CNS neurons which do not express ionotropic glutamate receptors or the HT22 hippocampal nerve cell line are exposed to millimolar glutamate concentrations, which are readily attainable within the damaged nervous system [44, 45], the cells die within 15 h. By 10 h, the endoplasmic reticulum and Golgi are swollen and are associated with large cytoplasmic vacuoles. Similarly, the mitochondria are swollen and show a loss of cristae. In contrast, the nuclear morphology remains normal, with no evidence of chromatin condensation, a major defining characteristic of apoptosis [30], (see also [28, 46]).

In addition to morphological studies, the glutamate-induced death of these cells has been studied at the biochemical level. Figure 3 outlines the known sequence of events which occurs in the presence of exogenous glutamate in HT22 cells; essentially all of these steps are also required for the death of mouse and rat cortical neurons by oxidative glutamate toxicity. Following exposure of the cells to glutamate, there is a rapid decline in the intracellular antioxidant GSH. If GSH reaches levels below 20% of normal for more than a few hours, the cells initiate a cell death program [47]. If glutamate is removed after shorter periods of GSH depletion, the cells recover. Therefore the nerve cells have a developed mechanism which allows them to be severely stressed (via depletion of GSH) and still survive for short periods of time. Associated with the initial trigger of GSH depletion, at least four other events must occur more or less simultaneously for the initiation of further downstream events which lead to cell death: (i) new messenger RNA (mRNA) and protein synthesis [29, 30]; (ii) the activation of one or more caspases [29, 30]; (iii) the activation of 12-lipoxygenase (12-LOX) [47]; (iv) the influx and metabolism of monoamines [48]. The requirement for protein synthesis and caspase activation occurs at the same time as GSH is being depleted [29], suggesting that the initiation of a decline in GSH levels may be a trigger for the activation of caspase and gene transcription. Little is known about this coupling mechanism at present. The coupling between 12-LOX activation and endogenous GSH depletion is, however, better understood. LOXs are dioxygenases which incorporate molecular oxygen into polyunsaturated fatty acids; 12-LOX, defined by the position of oxygen insertion in the fatty acid, is the predominant CNS form of the enzyme. The major product of 12-LOX in the brain is 12-hydroxyeicosatetraenoic acid (12-HETE). HETE's may play major roles in the modulation of synaptic transmission, and they also activate soluble guanylate cyclase (sGC) via a NO-independent pathway. Li et al. [47] showed that in cortical neurons and HT22 cells the depletion of GSH directly causes the translocation of 12-LOX to the membrane and activates 12-LOX enzymatic activity; inhibitors of 12-LOX block cell death. It was also shown in cell lysates that high concentrations of GSH inhibit 12-LOX activity. The products of 12-LOX activation have at least two functions; the activation of sGC to produce cyclic GMP (cGMP), which in turn activates a channel required for Ca^{++} influx late in the cell death pathway, and a LOX metabolite is required for the very large increase in the production of ROS which occurs around 6 h after the addition of glutamate to cells (fig. 3).

During the first 5 h after the addition of glutamate, there is about a 10-fold linear increase in ROS, which is followed by an exponential increase of over 200-fold relative to baseline [29]. While the initial linear increase in ROS is contemporaneous with the decline in GSH, the large increase only occurs after GSH is depleted below 20% of controls. In addition, the large increase in ROS production requires new mRNA and protein syn-

thesis, caspase activation, 12-LOX activation and an influx of monoamines [29, 30, 47, 48] since the inhibition of any one of these events blocks ROS accumulation and subsequent cell death. The massive increase in ROS is derived from mitochondria, for it can be directly inhibited by FCCP, an uncoupler of oxidative phosphorylation, and the complex III inhibitor antimycin A [29]. ROS production is also inhibited by the NADPH oxidase inhibitor diphenylene iodonium (DPI) and by concentrations of the monoamine oxidase inhibitor clorgyline, which are much higher than required for the inhibition of classical mitochondrial monoamine oxidase [48]. Both compounds do, however, react with a variety of flavoproteins, probably including those involved in the mitochondrial electron transport chain. In HT22 cells, exposure to glutamate leads to mitochondrial membrane hyperpolarization [S. Tan, unpublished]. Although mitochondrial membrane depolarization is usually associated with apoptosis, hyperpolarization can result if ADP is limiting, resulting in the inability of ATP synthase to use the electrochemical potential across the membrane. This condition promotes ROS production by mitochondria. Together, these data suggest, but do not rigorously prove, that the source of the massive burst of ROS following glutamate exposure is the mitochondria. Is it, however, these ROS which directly kill the cells, as is argued in the case of necrosis?

It is frequently assumed that in necrosis there is a large burst of ROS, rapid energy depletion or some other catastrophic metabolic event, which directly kills the cell. This is, however, probably not a valid assumption, and with HT22 cells and cortical neurons exposed to oxidative stress, it is clearly not the case. This is because cell death can be inhibited by blocking the necessary biochemical steps downstream of ROS accumulation,

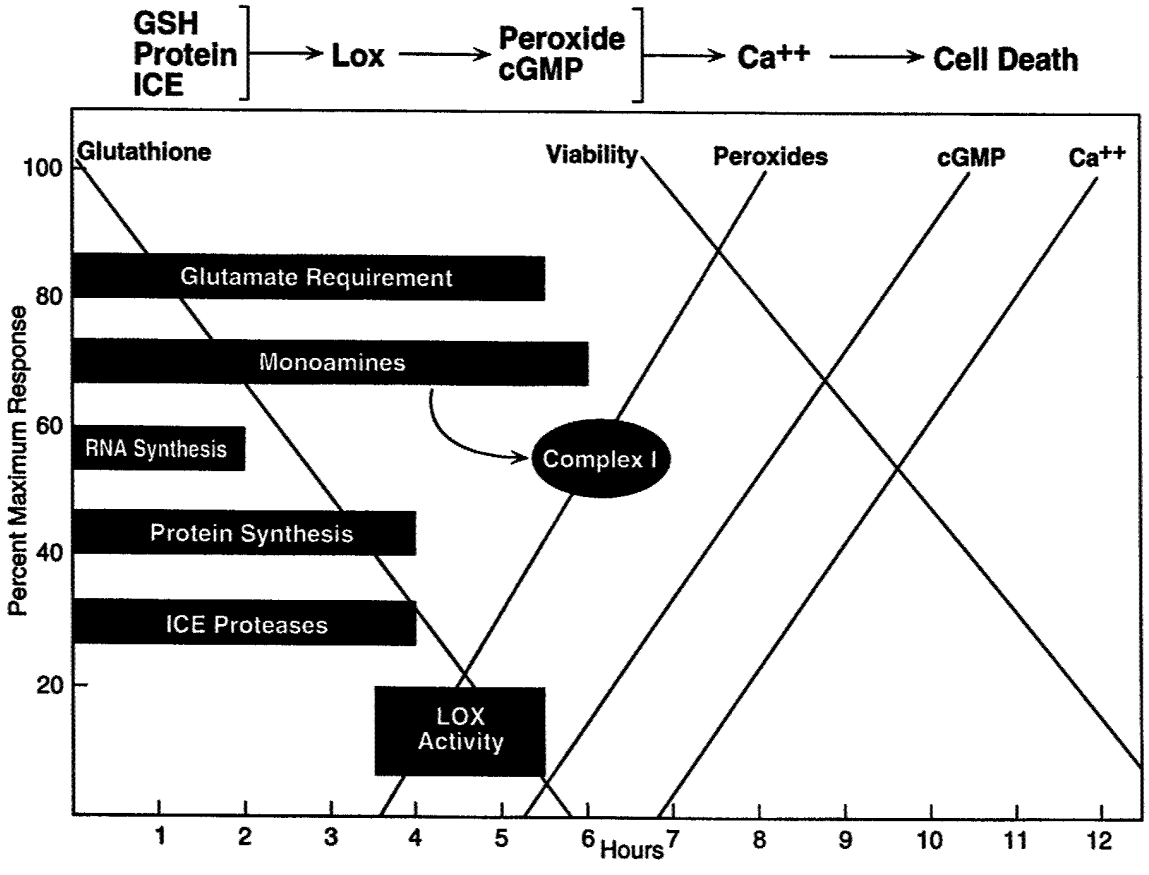

Figure 3. Schematic diagram of the time course of glutamate-induced cell death in rodent cortical neurons and the HT22 hippocampal nerve cell line. The solid lines indicate the relative increases or decreases in the molecules indicated and the solid rectangles indicate the times following the addition of exogenous glutamate when the indicated processes (e.g. RNA synthesis) occur. Monoamines are required for complex I in the mitochondria to generate ROS.

leaving cells viable for many hours in the presence of ROS at concentrations several hundredfold higher than normal—a condition most would view as catastrophic. The major downstream event from ROS accumulation which is required for cell lysis is Ca^{++} influx, which is to date the event closest in time to actual cell lysis which has been identified in oxidative glutamate toxicity. Ca^{++} influx occurs through a cGMP-activated Ca^{++} channel, and is required for cell death since death can be blocked by both cobalt, a nonspecific Ca^{++} channel inhibitor, and the removal of extracellular calcium. The function of this channel with respect to both Ca^{++} influx and cell death is also blocked by sGC inhibitors and inhibitors of ROS production [49]. Therefore, the accumulation of very large amounts of intracellular ROS is not sufficient to cause cell death, but it is a necessary step in the cell death process.

Finally, as stated above, in the oxidative glutamate toxicity cell death pathway there is no apparent decrease in mitochondrial membrane potential before complete failure, nor is there a release of cytochrome c. These data are at odds with most published accounts of apoptosis which show that there is a decrease in mitochondrial membrane potential. This membrane potential is established by the asymmetric distribution of protons and other ions across the inner mitochondrial membrane, and its loss frequently is associated with an irreversible commitment to cell death [50]. It has frequently been argued that the loss of the mitochondrial membrane potential is triggered by the sudden permeability increase of the inner mitochondrial membrane (permeability transition, PT), resulting in the leakage of proteins and the generation of ROS [51]. However, this does not occur in oxidative glutamate toxicity, again demonstrating the great diversity of mechanisms used by cells to carry out death programs.

ROS as signaling molecules

Although ROS have the potential to damage various intracellular macromolecules such as proteins, lipids and DNA, they also induce a variety of signaling pathways (for reviews see [52–56]). Whereas much of the research on these signaling pathways has been directed towards documenting a role for ROS in cell death (see earlier sections), there is also evidence that ROS can activate signaling pathways that lead to the induction of cell proliferation (e.g. [57–60]). The degree of overlap between the signaling pathways activated by pro-growth and pro-death conditions is not clear at this time. However, in general the activation of any type of signaling pathway by ROS involves the direct modification of a protein or other element of the signaling pathway by ROS. As described below, the consequences to cellular function may involve both the reversibility of the reaction and the location within the cell in which it occurs. In the following paragraphs a number of the proteins and signaling pathways affected by ROS will be described and the possible consequences of the modification of these pathways by ROS will be discussed. Studies which have utilized neurons or glial cells are specifically indicated. However, many of the studies utilized other types of cells, and effects of ROS on these proteins and signaling pathways in the cells of the nervous system remain to be determined.

Many of the effects of ROS may be mediated by reversible effects on intracellular proteins which lead to alterations in intracellular signaling pathways. Since the main intracellular antioxidants such as glutathione and Trx are thiol-containing molecules or proteins whose antioxidant activity is mediated by the reversible oxidation/reduction of cysteine sulfhydryl groups, it is likely that many of the direct effects of ROS on proteins are mediated by sulfhydryl groups in these proteins. For example, H_2O_2, which has been implicated in both oxidative stress and cell signaling, is a relatively mild oxidant that can oxidize protein sulfhydryl groups to produce cysteine sulfenic acid (CysS-OH) or disulfide bonds, both of which can be readily reduced back to cysteine by various intracellular reductants. However, under more severe and/or prolonged oxidative conditions sulfenic acid can undergo further oxidation to either sulfinic ($CysS\text{-}O_2H$) or sulfonic ($CysS\text{-}O_3H$) acid, both of which are irreversible. Despite the presence of cysteine in large numbers of proteins, only certain proteins are likely to be affected by this process since the oxidation of a protein sulfhydryl by H_2O_2 is dependent upon the pK_a of the target cysteine. For oxidation to occur, the pK_a of the target cysteine must be below 7.0, whereas the pK_a values of most cysteine resides in proteins is greater than 8.0 (see [54], and references therein). Although protein methionine residues can also be reversibly oxidized by H_2O_2, at the present time this amino acid has not been identified as playing a critical role in protein signaling. In addition, H_2O_2 and other oxidants may affect essential metal ions in specific proteins, changing their oxidation state and thereby altering their interaction with the protein. However, currently there is little direct evidence for this mode of action.

As discussed below, certain proteins such as protein tyrosine phosphatases and thioredoxin contain essential cysteine groups in their active sites which have low pK_a's, and their activities have been shown to be regulated by the cellular redox status. As more structural information becomes available regarding proteins involved in various signaling cascades, it should be possible to identify additional proteins whose activity is regulated by oxidation/reduction. These proteins are

likely to play important roles in the various responses of cells to changes in their redox status, and a characterization of their responses to oxidation could help define pathways involved in various aspects of cellular metabolism, including the response to oxidative stress.

Effects of ROS on protein kinase activity

ROS have been shown to increase the activity of both protein tyrosine kinases (PTKs) and serine/threonine kinases. However, in many cases it is not clear whether this is a direct effect on the kinase itself, an effect on an upstream activator or an effect on downstream protein phosphatases (see below). Since several recent reviews contain extensive lists of protein kinases which can be activated by ROS [55, 56, 61], we will focus instead on representative examples from each class of protein kinase and within a class, each potential mode of activation. Both receptor and nonreceptor PTKs can be activated by ROS. In both cases, the activation appears to be due mainly to an effect on downstream phosphatases (see below). For example, the treatment of cells with a variety of agents, including oxidants such as H_2O_2, was shown to induce tyrosine phosphorylation of the EGF receptor (EGFR) and PDGF receptor (PDGFR) by a mechanism involving the inhibition of dephosphorylation [62] rather than a direct effect on kinase activity. Indeed, treatment with H_2O_2 induced preferential phosphorylation of the EGFR on tyrosine, whereas ligand-activated receptor shows tyrosine, serine and threonine phosphorylation [63]. Furthermore, in many of these studies, whereas the phosphorylation of the receptors appeared to result in functional activation as determined by the binding of downstream substrates to the receptor, the actual effects of phosphorylation on receptor kinase activity were not determined.

A number of nonreceptor tyrosine kinases such as $pp60^{src}$ [64] and $p56^{lck}$ [65, 66] are shown to be activated by treatment of cells with H_2O_2 or other oxidants as determined both by increases in protein tyrosine phosphorylation and in vitro kinase activity. However, in the case of $p56^{lck}$, this activation could not be mimicked by direct treatment of the kinase with H_2O_2 [65], suggesting that the effects of ROS are indirect and probably mediated through the inhibition of phosphatase activity. In contrast, we have detected a direct effect of H_2O_2 on $pp60^{src}$ activity in immunoprecipitates, suggesting that in some cases ROS may be able to directly affect tyrosine kinase activity [unpublished]. Indeed, a number of years ago, Ltk, a nonreceptor, membrane-associated tyrosine kinase was described [67] which localizes to the endoplasmic reticulum and whose activity appeared to be regulated by the redox status of the cell. Dimerization through oxidation of specific sulfhydryl groups led to a significant activation of the kinase as determined by both in vitro kinase assays and anti-phosphotyrosine Western blots. Although this result could be due to an effect on phosphatases, the association of the majority of kinase activity with the dimerized protein, along with the lack of activation of the EGFR family member Neu under the same conditions, suggests that this tyrosine kinase may indeed be directly regulated by ROS. There is also some evidence that the PDGFR may be regulated by the redox status of the cell. Rigacci et al. [68] showed that the cellular GSH level specifically affected the tyrosine phosphorylation of the PDGFR in fibroblasts. These effects of ROS on PTK phosphorylation and/or activity may be cell type dependent. For example, H_2O_2 was found to specifically induce tyrosine phosphorylation and activation of $p72^{syk}$ in B lymphocytes, whereas in contrast to the studies described above, it had no effect on $p56^{lck}$ [69]. Treatment of cells with H_2O_2 also stimulates the tyrosine phosphorylation of the focal adhesion kinase, $pp125^{FAK}$. However, in contrast with the other PTKs discussed above, the phosphorylation of this kinase does not correlate with its activation. Furthermore, the phosphorylation of $pp125^{FAK}$ was not inhibited by several different PTK inhibitors [70]. For the most part, the consequences for the cell of PTK activation by oxidants is not clear. However, at least in some cases it appears to be part of a protective response. For example, ultraviolet (UV) irradiation of HeLa cells results in the ROS-dependent activation of the nonreceptor PTK $pp60^{src}$, whereas inhibition of $pp60^{src}$ activity potentiates cell killing by UV [64].

ROS also affect the activities of serine/threonine kinases. Perhaps the best known of these are members of the mitogen-activated protein kinase (MAP kinase) family. These serine-threonine kinases are activated by dual phosphorylation in response to a variety of extracellular stimuli. In mammalian cells, at least three distinct members of the MAP kinase family are expressed: ERKs (also known as MAPKs), stress-activated protein kinase (also known as c-Jun NH2-terminal kinase (JNK)) and p38 MAPK. ERKs are activated by growth factors and are primarily involved in cell proliferation and differentiation, whereas JNK and p38 MAPK are primarily activated in response to proinflammatory cytokines and environmental stress and are implicated in inflammatory responses, cell cycle arrest, DNA repair and cell death. The standard pathways for activation of these protein kinases consist of two upstream protein kinases: MAPKKKs, which phosphorylate and thereby activate MAPKKs, which phosphorylate the MAPKs on both a threonine and a tyrosine residue (for reviews see [71–74]). All three members of this family, including ERKs [75, 76], can be specifically activated by H_2O_2 and other oxidants, as determined both by dual phos-

phorylation and in vitro kinase assays. The specific ROS which lead to ERK activation may be cell type dependent since, in some cases, activation is seen with H_2O_2 [75], whereas in others it is seen with superoxide but not H_2O_2 [76]. ERK activation by H_2O_2 appears to be indirect since it is blocked by a dominant-negative Ras mutant, suggesting that H_2O_2 activates an upstream member of the prototypical Ras-Raf-MEK-MAPK pathway. Indeed, some reports suggest that Ras is responsive to the redox status of the cell and can be directly activated by ROS such as H_2O_2 [77] or nitric oxide [78]. A role for ERK activation in inhibiting cell death induced by a variety of stimuli has been demonstrated in a number of studies in a variety of different cell types including neuronal cells (e.g. [75, 79–82]).

Another, recently described member of the MAPK family, ERK5 or BMK1, is also activated by ROS [83] but through a very different pathway. In the case of ERK5, the activity of the PTK $pp60^{src}$ appears to be essential for ERK5 activation by H_2O_2 [83]. Other members of the src family of PTKs cannot substitute for $pp60^{src}$. Interestingly, in apparent contrast to the other members of the src family, $pp60^{src}$ can be directly activated by H_2O_2 (see above).

Several alternative mechanisms have been identified for the activation of the two other members of the MAPK family, JNK and p38 MAPK, by ROS. For example, JNK is generally thought to be activated through a pathway involving small GTP-binding proteins and a series of protein kinases terminating in the dual specificity kinases MKK4 and/or MKK7. However, recently Meriin et al. [84] have demonstrated that certain stimuli, including oxidative stress induced by a variety of agents, activate JNK not through this cascade but via inactivation of the phosphatases which generally maintain this kinase in a dephosphorylated and, therefore, inactive state. A second mechanism for the activation of both JNK and p38 MAPK by oxidative stress has also been described. ASK1 is a MAPKKK which can stimulate pathways leading to both p38 and JNK activation [85]. ASK1 activation is implicated in the promotion of programmed cell death [85, 86]. ASK1 activity in turn is regulated by the redox status of the cell. Two mechanisms for this regulation have been described. In one case [86], activation of ASK1 was brought about by H_2O_2-induced dimerization of the protein. Similar activation could be induced by synthetic dimerization. In the second study [85], activation of ASK1 was dependent upon Trx, a redox-sensitive regulatory protein (for review see [87]). Trx was found to associate with the N-terminus of ASK1 in a redox-sensitive fashion, resulting in inactivation of the kinase. Treatment with H_2O_2 or other oxidants resulted in the oxidation of Trx and its release from ASK1, leading to the activation of JNK and p38 MAPK. These two observations may not be at odds if the loss of Trx binding leads to the dimerization of ASK1. However, the role of ASK1 in either JNK or p38 MAPK activation may be cell type dependent since it is not the major MAPKKK for these kinases in many types of cells. A third mechanism for the regulation of JNK activity involves yet another protein involved in the maintenance of the cellular redox potential. Recently, glutathione *S*-transferase Pi (GSTp) was identified as a specific inhibitor of JNK [88]. An increase in ROS levels in cells led to GSTp oligomerization, dissociation of the GSTp-JNK complex and an increase in JNK activity. Furthermore, fibroblasts from GSTp-null mice had high levels of basal JNK activity relative to their normal counterparts which could be reduced by transfection with GSTp. In contrast with ERKs, the role of JNK in programmed cell death is controversial. Initial evidence suggested that high levels of JNK activity contributed to cell death, whereas inhibition of JNK activation was protective [79]. However, later studies have demonstrated a protective role for JNK activation in several different cell death paradigms (e.g. [89, 90]). One complicating factor is that the JNK family is encoded by three different genes (*Jnk1*, *Jnk2*, *Jnk3*) with alternative splicing giving rise to 10 different isoforms (4xJNK1, 4xJNK2, 2xJNK3) (for reviews see [72, 73]). A number of studies suggest that the different JNK isoforms may not be functionally redundant within cells. For example, recent studies with mice deficient in JNK1, JNK2, JNK3, or several combinations thereof, indicate distinct roles for the different isoforms in neuronal programmed cell death [91]. Whereas JNK3 knockout mice show an increased resistance to kainic acid-induced seizures and subsequent programmed cell death of hippocampal neurons, mice deficient in both JNK1 and JNK2 have a severe dysregulation of neuronal programmed cell death during embryonic development. Specifically, whereas these mice show a decrease in neuronal programmed cell death in the hindbrain, greatly increased neuronal programmed cell death is observed in the forebrain. The role of p38 MAPK in programmed cell death is slightly less controversial. Using specific p38 MAPK inhibitors, a number of studies have demonstrated that inhibition of p38 MAPK activity promotes cell survival in both neuronal [92, 93] and nonneuronal [94, 95] cells following exposure to a variety of stressful conditions.

The PKC family is a heterogeneous group of phospholipid-dependent serine/threonine kinases that are thought to be key elements in signaling pathways which regulate a wide range of cellular functions, including cell survival. The rapid loss of PKC activity is considered a prognostic feature of lethal damage to neurons following ischemia in vivo and hypoxic and excitotoxic insults in vitro (for reviews see [96–99]). The PKC family at present contains 11 different members which

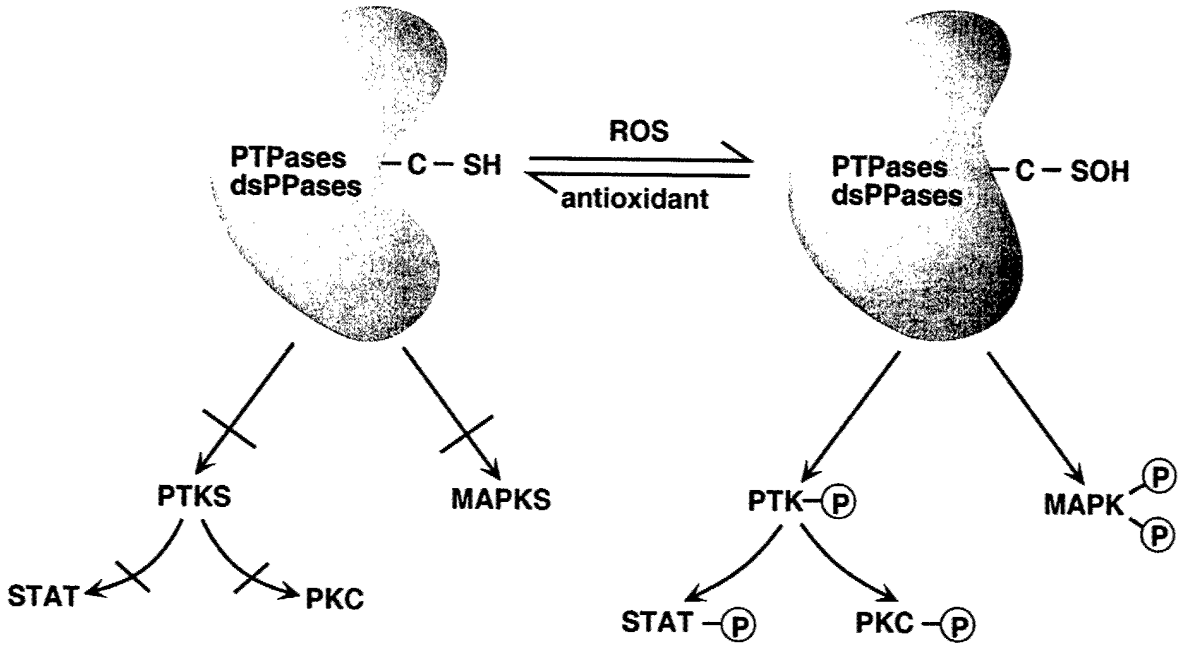

Figure 4. Regulation of protein phosphatase activity by H_2O_2. Both protein tyrosine phosphatases (PTPases) and dual specificity phosphatases (dsPPases) can be reversibly inactivated by H_2O_2 through the covalent modification of an essential cysteine in the active sites of the enzymes. Normally, PTPase activity maintains protein tyrosine kinases (PTKs) and PKCs, as well as members of the STAT family of transcription factors, in a state of low phosphorylation which, in the case of PTKs, correlates with low activity. Similarly, both dsPPase activity and PTPase activity maintain MAPKs in an unphosphorylated or partially phosphorylated state, which is inactive. Inhibition of phosphatase activity by H_2O_2 results in the activation of these proteins through autophosphorylation and/or phosphorylation by upstream kinases.

can be divided into three groups on the basis of structure and cofactor requirements. All members of this protein family are both phosphorylated on tyrosine and activated by treatment of cells with H_2O_2 and other ROS [100]. The effect of ROS on PKC appears to be specific to this protein kinase family because other, related protein kinases were not phosphorylated by the same treatments. Although the effect of ROS on PKC is clearly indirect, it is not clear whether it is mediated by activation of upstream PTKs or inhibition of downstream phosphatases, or both, nor is it clear what the consequences are to overall cellular function.

Effects of ROS on protein phosphatases

As alluded to in the section on protein kinases, a major mechanism for the activation of protein kinases by ROS may be through the inhibition of specific phosphatases (fig. 4). This is because kinases are activated by phosphorylation, and since phosphatases generally have about 10 × faster reaction kinetics than kinases, normally the dephosphorylated and therefore inactive state of the kinase predominates. There are three major classes of phosphatases in cells: tyrosine phosphatases (PTPases), serine/threonine phosphatases (PPases) and dual specificity (Thr/Tyr) phosphatases (dsPPases). The best-understood effects of ROS on phosphatase activity are on the PTPases. Within their active sites the PTPases contain a cysteine residue which is essential for enzymatic activity because it participates directly in the dephosphorylation reaction [101]. This cysteine is also very sensitive to the redox status of the cell. Although indirect evidence in a number of studies suggested that PTPases are a major intracellular target of H_2O_2 and other ROS (e.g. [62, 102]), recent work in the laboratory of Denu [103] directly demonstrated that a variety of different PTPases could be directly and rapidly inactivated by treatment with low concentrations of H_2O_2. This treatment resulted in the conversion of the essential catalytic cysteine to a stabilized sulfenic acid intermediate. Although this reaction is readily reversible in the presence of reducing agents, the reactivation process is much slower than the inactivation process, so that inactivation can occur in the presence of reducing conditions such as might be present in the cell.

dsPases act specifically on the terminal members of the MAPK family (e.g. ERKs, JNKs and p38 MAPKs) (for review see [104]). They also contain an essential cysteine in their active sites so they would be expected to undergo the same type of regulation in response to H_2O_2 or other oxidants as PTPases. However, since the activity of each of the substrates of these phosphatases is dependent upon phosphorylation of both tyrosine and threonine residues, these kinases can be inactivated by PTPases and PPases as well. Indeed, there is good evidence that in vivo PTPases play a role in regulating MAPK activity [104]. The evidence for a role for PPases in regulating MAPK activity is more controversial. However, in a recent paper it was demonstrated that p38 MAPK activation by H_2O_2 in astrocytes was concurrent with the inhibition of PPase activity [105]. In addition, direct inhibition of a subset of PPases (PP1 and PP2A) by okadaic acid also resulted in activation of p38 MAPK, whereas inhibition of another PPase, calcineurin (PP2B), did not.

Unlike the PTPases, PPases do not contain an essential cysteine residue at their active sites, so that the mechanisms underlying their inactivation by ROS are likely to be different. Indeed, even which ROS can and cannot inactivate these phosphatases is unclear. The Denu laboratory [103] found that the same conditions that resulted in complete inactivation of PTPases had no effect on the activities of three distinct PPases, PP2C, λ phosphatase and calcineurin. However, an earlier study had found that SOD could protect calcineurin from inactivation [106] and was thought to do so by blocking the oxidation of iron in the active site of calcineurin by $O_2^{\cdot -}$. Similar to the other PPases, calcineurin contains a pair of metal ions at the active site which are important for both catalytic activity and the structural integrity of the phosphatase [101]. $O_2^{\cdot -}$ may cause oxidation of the iron group in the active site, thereby rendering the phosphatase inactive. Very recently, it was shown that calcineurin can also be reversibly inactivated by H_2O_2 [107]. This inactivation does not involve the metal ions at the active site but rather appears to result from the cross-linking of two cysteines which lie outside the active site. How this cross-linking results in inactivation of calcineurin remains to be determined. In addition, both PP1 and PP2A are inactivated by reaction with GSSG [108]. The production of this oxidized form of glutathione is dependent upon the redox status of the cell [109]. Since GSSG interacts with cysteine groups in proteins [110, 111], these data suggest that a cysteine outside the active site but within the catalytic domain of these phosphatases is critical for enzyme activity and can be reversibly modified in a manner dependent upon the redox status of the cell. Thus, while PTPases and dsPPases can be directly affected by ROS such as H_2O_2, the PPases may be affected both directly via their metal ions and indirectly via redox-dependent amino acid modifications. These findings suggest that the spectrum of phosphatase inactivation by ROS within a given cell type will depend upon both the specific oxidizing agent and the effects that this agent has on the various detoxifying systems within the cell. The ultimate consequences of phosphatase inactivation by ROS on cellular function are not clear at this time but are likely to be cell type specific since they will depend upon the spectrum of kinase pathways which are activated or inhibited.

Activation of transcription factors by ROS

A number of different transcription factors are activated by ROS. Surprisingly, rather than a single mechanism for activation, a range of activation mechanisms have been identified. These include both direct effects on the transcription factors themselves and indirect effects mediated by either regulatory binding partners or upstream signaling pathways. Furthermore, at least in some cases, the activation is specific for a single type of ROS. The following paragraphs detail a number of these activation mechanisms and the transcription factors that they affect.

The signal transducer and activator of transcription (STAT) factors were originally described as growth factor- and interferon-inducible DNA binding complexes (for reviews see [112, 113]). These transcription factors play a role in the regulation of many genes, including the c-fos protooncogene, caspases and the cell cycle regulator $p21^{CIP1/WAF1}$. Unlike other transcription factors, the STAT factors are phosphorylated on tyrosine residues when cells are treated with an appropriate stimulus. Following phosphorylation the STATs undergo homo- and/or heterodimerization via SH2-phosphotyrosine interactions and become competent to bind DNA. The STATs also translocate from the cytoplasm to the nucleus following activation. Several PTKs have been identified, including Janus kinases (JAKs) and Tyk, which can phosphorylate STATs. Recently, several groups showed that along with cytokines and growth factors, STATs could also be activated by ROS [114, 115]. In one study using rat-1 fibroblasts, STAT3 was shown to be specifically activated, as determined by both DNA binding activity and transcriptional activity, by H_2O_2 but not by agents which generate either superoxide or NO [115]. This induction was blocked by antioxidants. The activation appeared to be via the upstream tyrosine kinases JAK2 and Tyk, whose activity was also induced by H_2O_2. However, the mechanism for this induction is still unclear since whereas direct inhibition of PTPases can strongly activate STATs, the pattern of activation is quite different from that seen

with H_2O_2 [115]. However, it maybe that H_2O_2 can only inhibit a subset of PTPases which act on STATs and/or their upstream kinases. In the second study which used lymphocytes [114], STAT3 was also found to be specifically activated by H_2O_2, and this activation was enhanced in the presence of PTPase inhibitors. Intriguingly, phenanthroline, an iron chelator, also blocked STAT activation by H_2O_2, suggesting that hydroxyl radicals could play a role in this activation. Thus, ROS appear to selectively activate a specific member of the STAT family by a mechanism which involves the activation of upstream activating kinases. The effects of STAT3 activation by ROS on cellular function remain to be elucidated.

The ARE or antioxidant responsive element is found in the promoters of a battery of genes which encode antioxidant and detoxification proteins and is responsible for the coordinated transcriptional activation of these genes. Among the genes which are activated through the ARE are enzymes which can correct imbalances in the cellular redox state resulting from decreases in GSH and/or increases in ROS. Included in the five classes of compounds which act on this promoter are both redox-cycling agents such as quinones and prooxidants such as H_2O_2 and lipid hydroperoxides. The transcription factors which act on this promoter and a partial picture of the mechanism underlying the activation of these factors have been recently elucidated (for reviews see [109, 116]). The transcription factor which binds and activates the ARE is a heterodimer of a small Maf protein and Nrf2. Maf is required for the sequence-specific DNA binding of the factor, whereas Nrf2 induces the transcriptional activation. The ability of Nrf2 to induce transcriptional activation is regulated by agents which activate the ARE, indicating that Nrf2 is directly involved in transducing the ROS signal. In unstimulated cells Nrf2 is found predominantly in the cytoplasm bound to a recently identified protein called Keap1 which, by interacting with the cytoskeleton, appears to keep Nrf2 from migrating to the nucleus [117]. Upon stimulation, the repression of Nrf2 by Keap1 is released, and Nrf2 translocates to the nucleus where it can interact with Maf and induce activation of the ARE. Exactly how ROS or other activators release the repression of Nrf2 by Keap1 is not clear, nor is it clear whether Nrf2 migrates to the nucleus along with Keap1 or whether activation releases Nrf2 from Keap1.

The regulation of the transcription factor AP1 by ROS is quite complex and highly dependent upon the context in which the regulation is examined. However, the studies with this transcription factor provide an excellent example of some of the confusion in the field as well as the way that the experimental paradigm affects the outcome. In addition, recent work on the regulation of AP1 by the intracellular redox potential points to a novel mechanism for transcription factor regulation whose importance in overall protein regulation is only beginning to be recognized. More details on AP1 regulation by ROS can be found in a number of recent reviews (e.g. [55, 61, 118, 119]).

Unlike the other transcription factors discussed here, AP-1 is not a single transcription factor but rather a group of related, dimeric complexes composed predominantly of Jun homodimers and Jun-Fos heterodimers (for reviews see [120–122]). Both the Jun and Fos families contain multiple members resulting in quite a complicated group of transcription factors. The differences in the different complexes are not well understood, although there is evidence that different complexes show distinct effects on transcriptional regulation. Dimerization of these proteins occurs via leucine zipper domains and results in the formation of a bipartite DNA binding site. Dimerization is necessary but not sufficient for DNA binding, which is mediated by amino acids in the adjacent basic region of the proteins. The dimers interact with the tetradecanoic phorbol acetate (TPA)-response elements in the promoter regions of a wide variety of genes implicated in cell proliferation, tumor promotion and the cellular response to stress. Similar to the other transcription factors discussed here, the DNA binding domains and the transcriptional activation domains of Jun and Fos reside in distinct regions of the protein. Curiously, the activation domains of these two proteins are controlled by different signaling pathways. The activation domain of Jun is regulated predominantly by the JNK kinases (see above), which phosphorylate Jun at serines 63 and 73 by a two-step mechanism wherein JNK first associates with Jun and subsequently phosphorylates it. As discussed in the previous section on ROS and protein kinases, JNK kinase activity is regulated by ROS. Although the Fos activation domains are also regulated by phosphorylation, the kinases involved remain to be identified. However, there is evidence indicating that the transcriptional activity of AP-1 complexes can also be influenced by signaling through the ERK pathway, which is also regulated by ROS (see above) [122]. Interestingly, genetic analysis indicates that both c-Jun and c-Fos null mice have increased levels of cell death following UV treatment as compared with their wild-type counterparts. This suggests that AP-1 activation results in the synthesis of proteins which are important in protecting cells from UV-induced cell death [122]. However, other studies have suggested that AP-1 activation can promote cell death (e.g. [120, 121]). Thus, similar to the role of JNK activation in programmed cell death, the role of AP-1 activation is open to debate. The AP-1 complex is also regulated by the expression of the Fos and Jun proteins. However, the relationship between protein expression and transcriptional activa-

tion is not straightforward. Whereas H_2O_2 strongly induces Fos and Jun expression, it only weakly induces AP-1 activity as defined by both DNA binding and transcriptional activation. In contrast, antioxidants strongly induce both protein expression and AP-1 activity [118, 123].

Thus, it is clear from the above description that ROS can affect AP-1 activity at a number of different sites ranging from induction of the synthesis of Fos or Jun to controlling protein phosphorylation to regulating DNA binding. In whole cells, treatment with H_2O_2 or agents which produce ROS can result in the activation of AP-1 (see [55, 118, 123]). However, numerous studies have shown that in vitro ROS inhibit DNA binding by AP-1. The redox regulation of DNA binding by AP-1 was first noted 10 years ago by Curran et al. [124]. They showed that a conserved cysteine in the DNA binding domain of Fos and Jun could be reversibly oxidized, resulting in a complex with little or no DNA binding activity. Mutation of the cysteine to serine resulted in an increase in DNA binding activity and a loss of redox regulation. Interestingly, the oncogenic homologue of c-Jun, v-Jun, already has this substitution, suggesting that part of its transforming potential may be due to a loss of redox regulation. Recent studies on the nature of the oxidized cysteine from Lamas et al. [125] has uncovered a novel mode of redox regulation for AP-1. They set out to examine whether the DNA binding activity of c-Jun could be regulated by the ratio of reduced to oxidized GSH. In normal cells, the ratio exceeds 100, whereas in various models of oxidative stress the ratio drops to values between 10 and 1. Half-maximal inhibition of AP-1 DNA binding activity was found at a GSH/GSSG ratio of 10 and was shown to be due to the reversible S-glutathiolation of the conserved cysteine in the DNA binding domain of c-Jun. Although a reduction in the GSH/GSSG ratio also caused c-Jun dimerization through a conserved cysteine in the leucine zipper region of the protein, this dimerization did not affect DNA binding. Several mechanisms for mixed disulfide formation with GSH have been proposed [110, 111], but further work will be required to define the mechanism operating here. Interestingly, mixed disulfide formation while temporarily inhibiting DNA binding activity could have the long-term effect of protecting the protein against oxidative damage by preventing the irreversible oxidation of cysteines to sulfinates and sulfonates. The oxidation may be reversed by Ref1. Ref1 is a DNA repair enzyme which can also regulate AP-1 activity [61, 119] and is probably the AP-1 activity protein originally described by Curran et al. [124]. The reducing activity of Ref1 is regulated by Trx (see above).

NF-κB was one of the first transcription factors shown to be regulated by ROS and is often considered to be a primary sensor of oxidative stress in cells (for reviews see [3, 119, 126]). Furthermore, in neuronal cells, NF-κB is implicated in playing a critical role in resistance to oxidative stress [127]. NF-κB is a member of a family of proteins which can homo- and heterodimerize to form a complex capable of binding to DNA. The most common form of this complex is the p50/p65 heterodimer. In unstimulated cells, NF-κB is found in the cytoplasm associated with the inhibitory protein IκB. Although a number of IκB proteins exist, the regulation of IκBα is the best understood. Following treatment of cells with many different stimuli, IκB is phosphorylated by an IκB kinase (IKK) and subsequently degraded in a proteosome-dependent manner. The free NF-κB is now able to translocate to the nucleus where it can bind DNA and initiate transcription of a diverse array of target genes including cytokines and growth factors, oxidative-stress-related enzymes, antiapoptotic proteins and cell-adhesion molecules. Many treatments which activate NF-κB are known to increase the production of ROS, including UV light and TNF-α, and antioxidants generally block NF-κB activation by these treatments. In addition, direct addition of H_2O_2 to some, but not all, cell lines activates NF-κB. Further evidence for the critical role of ROS and specifically, H_2O_2, in NF-κB activation comes from studies on cells stably overexpressing either catalase or SOD [128]. While catalase blocked the appearance of the active form of NF-κB in response to treatment of cells with either TNF-α or okadaic acid, SOD did not. The effect of catalase overexpression could be reversed by the catalase inhibitor, 3-aminotriazole.

It is not known how ROS activate NF-κB. One pathway involves the degradation of IκB as described above, but it is not clear whether ROS directly or indirectly increase the activity of an IKK or, instead, inhibit the activity of an IκB phosphatase. A second pathway for NF-κB activation has also been described and may be the predominant pathway induced by some forms of oxidative stress. Tyrosine phosphorylation of IκB on specific residues can result in the release of IκB from NF-κB without the resulting degradation of IκB [129]. This mechanism appears to be particularly prominent following reoxygenation after hypoxia [129, 130]. Since oxidative stress can block PTPase activity (see above), it seems likely that this mechanism for NF-κB activation is due to the direct inactivation of a PTPase that normally maintains IκB in a dephosphorylated state. Indeed, it was shown that in Jurkat cells, NF-κB activation required the activity of $p56^{Lck}$ [129] whose activity is known to be indirectly enhanced by ROS through the inhibition of PTPases (see above).

Similar to AP-1, NF-κB in the nucleus requires a reducing environment for DNA binding activity. This suggests that the agents which induce oxidative stress and

NF-κB activation must do so locally within the cytoplasm and that severe oxidative stress which leads to oxidizing conditions within the nucleus as well as the cytoplasm might result in an inability of NF-κB to activate transcription.

In addition to the transcription factors discussed above, the activities of a number of other transcription factors have been shown to be regulated by the redox status of the cell (for reviews see [55, 61, 118, 123, 131]). In almost all the cases which have been examined, this regulation involves direct effects on critical cysteines in the DNA binding domains or transcriptional activation domains of the proteins. A number of these transcription factors show the same conflict as seen with AP-1 wherein ROS activate the transcription factor in whole cells but in vitro studies with the isolated factor show it to be inhibited by oxidation. Clearly, further studies are required to resolve this apparent contradiction. Furthermore, these types of results indicate that whole cell data are critical when evaluating the effects of ROS on a given transcription factor. Among the other factors which show this dichotomy are NF-κB and p53. Other transcription factors are inhibited by oxidation both in in vitro assays and in whole cells. These include Sp-1, Egr-1 and GR. Another set have only been examined in one or the other assay systems, including USF and Ets. Thus, ROS may play critical roles in regulating the activity of a large number of transcription factors. The precise consequences of an increase in ROS depend not only upon the nature of the transcription factor itself but also upon the conditions within a given cell. This is most apparent for those transcription factors which appear to require an oxidizing cytoplasm but a reducing nucleus. Subtle changes in this balance could dramatically affect the activity of these transcription factors.

Summary

In this review emphasis has been placed upon the role of ROS as agents which initiate specific signaling pathways rather than agents which nonspecifically and irreversibly damage intracellular macromolecules. Therefore, even when ROS production leads to cell death, this maybe due to the activation of one or more signaling pathways leading to metabolic changes in the cell which eventually bring about cell death, and not due to generalized cellular damage. In a given cell type, the same ROS can lead to a different series of downstream events depending upon the concentration of the ROS and the duration of exposure. Similarly, the delivery of prooxidant stress by different means also may not produce the same set of physiological responses, and the same concentration of ROS can have distinct effects in different cell types. It follows that attempts to pigeonhole the cellular response to a given ROS into one or two narrowly defined categories will inevitably lead to a great deal of confusion, and that each cellular response to a given stress must be considered unique until proven otherwise.

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Oxidative Glutamate Toxicity Can Be a Component of the Excitotoxicity Cascade

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Along with ionotropic and metabotropic glutamate receptors, the cystine/glutamate antiporter x_c^- may play a critical role in CNS pathology. High levels of extracellular glutamate inhibit the import of cystine, resulting in the depletion of glutathione and a form of cell injury called oxidative glutamate toxicity. Here we show that a portion of the cell death associated with NMDA receptor-initiated excitotoxicity can be caused by oxidative glutamate toxicity. In primary mouse cortical neurons the cell death resulting from the short-term application of 10 μM glutamate can be divided into NMDA and NMDA receptor-independent phases. The NMDA receptor-independent component is associated with high extracellular glutamate and is inhibited by a variety of reagents that block oxidative glutamate toxicity. These results suggest that oxidative glutamate toxicity toward neurons lacking functional NMDA receptors can be a component of the excitotoxicity-initiated cell death pathway.

Key words: excitotoxicity; brain; death; nerve; non-NMDA; oxidative stress

The physiological consequences of extracellular glutamate are mediated by three classes of membrane proteins within the CNS. These are ionotropic glutamate receptors, metabotropic glutamate receptors, and the cystine/glutamate antiporter. Ionotropic glutamate receptors have two known roles. They are responsible for the majority of excitatory neurotransmission and also for a great deal of CNS pathology. In cases of stroke or trauma, excessive extracellular glutamate leads to nerve cell death via the activation of NMDA receptors (Rothman and Olney, 1986). This phenomenon, which can be reproduced in cell culture (Rothman, 1985; Choi, 1987), is termed excitotoxicity (Olney, 1986). In contrast to ionotropic glutamate receptors, the metabotropic glutamate receptors (mGluRs) are G-protein-coupled membrane proteins with a wide variety of biological functions (Nakanishi, 1994). Finally, a third target for extracellular glutamate in the CNS is the inhibition of the glutamate/cystine antiporter x_c^-, which results in a form of oxidative stress and cell death called oxidative glutamate toxicity (Murphy et al., 1989). The glutamate/cystine antiporter couples the import of cystine to the export of glutamate (Sato et al., 1999). Concentrations of extracellular glutamate as low as 100 μM, which are well below the level of extracellular glutamate found in models of stroke and trauma (see, for example, McAdoo et al., 1999), completely inhibit the uptake of cystine (Sagara and Schubert, 1998). Cystine is required for the synthesis of the potent intracellular-reducing agent glutathione (GSH). When GSH is depleted by extracellular glutamate, cells die from a form of programmed cell death (Tan et al., 1998a,b).

The potential role of oxidative glutamate toxicity in ischemia and trauma is not understood, but there have been strong indications that several cell death pathways are involved. In localized cerebral infarction the neurons in the epicenter die rapidly, whereas those more distal remain viable for several hours (Siesjo, 1992). Multiple forms of nerve cell death also have been identified in excitotoxic CNS primary culture paradigms that follow exposure to glutamate (for review, see Choi, 1992). In primary cultures of cerebellar granule cells that are exposed to glutamate, there is a rapid necrotic phase, followed by delayed apoptotic-like cell death (Ankacrona et al., 1995). During oxygen–glucose deprivation of primary mouse cortical cultures or organotypic cultures of the rat hippocampus, some cell death occurs from ionotropic receptor-independent mechanisms (Gwag et al., 1995; Newell et al., 1995). All of these observations are consistent with *in vivo* data, which show that glutamate receptor-independent programmed cell death may occur after ischemic insults (Shigeno et al., 1990; Linnik et al., 1993; MacManus et al., 1993; Okamoto et al., 1993). In addition, animals that lack caspases undergo a form of cell death that is morphologically very similar to oxidative glutamate toxicity (Tan et al., 1998a,b; Oppenheim et al., 2001). A number of parameters change dramatically during CNS stress, leading to the observed high exogenous glutamate. These include the direct release of glutamate from cells, the enzymatic conversion of high extracellular glutamine to glutamate, and the shutdown of nerve and glial glutamate uptake systems by pro-oxidant conditions (see Discussion). It is therefore of interest to determine whether oxidative glutamate toxicity can play a significant role in nerve cell death that is associated with the excitotoxicity cascade.

MATERIALS AND METHODS

Cell culture. Primary cultures of cortical neurons that die reproducibly by excitotoxicity were prepared by combining aspects of two published protocols (Rose et al., 1993; Dugan et al., 1995). Embryonic day 14 (E14) BALB/c mouse embryo cortices were minced and treated with 0.1% trypsin for 20 min. After centrifugation the cells were resuspended in B27 Neurobasal medium (Life Technologies, Grand Island, NY) plus 10% fetal calf serum and were dissociated by repeated pipetting through a 1 ml blue Eppendorf pipette tip. Then the cells were plated at 1×10^5 cells per well in 96-well poly-L-lysine and laminin-coated microtiter plates (Becton Dickinson, Bedford, MA) in B27 Neurobasal plus 10%

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fetal calf serum and 20% glial growth-conditioned medium prepared according to Dugan and colleagues (Dugan et al., 1995). The growth-conditioned medium improved plating efficiency by ~30%. Then 2 d later the medium was aspirated and replaced by serum-free B27 Neurobasal medium plus 10 μg/ml cytosine arabinoside. The cultures were used without media change between 7 and 12 d after plating and were essentially free of astrocytes (Brewer et al., 1993).

For glutamate toxicity assays, test drugs (e.g., antioxidants) were added 30 min before glutamate exposure. Then the culture medium was moved with a multichannel pipette to a new 96-well plate, and the cells were exposed to glutamate (usually 10 μM) in a HEPES-buffered salt solution [HCSS (Rose et al., 1993)] containing (in mM) 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 15 glucose, and 20 HEPES, pH 7.4. In some cases, 1 μM glycine was included, but this had no net effect on excitotoxic death. After 10 min at room temperature the HCSS was aspirated, and the original growth medium was returned to the cells. In some cases the NMDA antagonist aminophosphonopentanoic acid (AP-5) was added at this point to inhibit the downstream activation of glutamate receptors.

MTT assay. Cell survival was determined by the MTT [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as described (Schubert et al., 1992), which correlates with cell death as determined by trypan blue exclusion and a colony-forming assay (Davis and Maher, 1994). At 24 hr after the addition of glutamate, 10 μl of the MTT solution (2.5 mg/ml) is added to each well and the cells are incubated for 3 hr at 37°C. Then 100 μl of solubilization solution (50% dimethylformamide and 20% SDS, pH 4.8) is added to the wells, and the next day the absorption values at 570 nm are measured. The results are expressed relative to the controls specified in each experiment. They are expressed as the mean of triplet determinations within the same experiment ± SEM; each experiment has been repeated at least three times with similar results.

Western blotting and glutamate assays. For Western blotting the cells were collected directly in Laemmli buffer (Laemmli, 1970). Cell lysates were resolved in 10% polyacrylamide gels containing SDS and transferred electrophoretically to hybridization membranes (Micron Separations, Westboro, MA). The membrane was probed first with a rabbit antiserum at a dilution of 1:2000 and then with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody at a dilution of 1:20,000. The antibody conjugates were detected with a chemiluminescence Western blot kit (Amersham, Buckinghamshire, UK).

Glutamate assays in growth-conditioned medium were performed by both mass spectroscopy and standard amino acid analysis with similar results (Iwabuchi et al., 1994). The standard amino acid data are presented. The removal of glutamate from growth-conditioned medium was done exactly as described by Matthews et al.(2000), except that the enzyme was added every 6 hr during the experiment. Media were treated initially for 1 hr at 37°C with 100 μg/ml glutamate pyruvate transaminase (GPT), 100 μM pyridoxal-L-phosphate and 10 mM pyruvate; these reagents were left in the culture medium.

Reagents. The mGluR agonists and antagonists were all from Tocris Cookson (Ballwin, MO), and mGluR 1 and 2/3 antisera and anti-NMDA antisera were from Chemicon (Temecula, CA). Anti-mGluR5 was a gift from Dr. R. Gereau (The Salk Institute, La Jolla, CA). The remaining reagents were obtained from Sigma (St. Louis, MO).

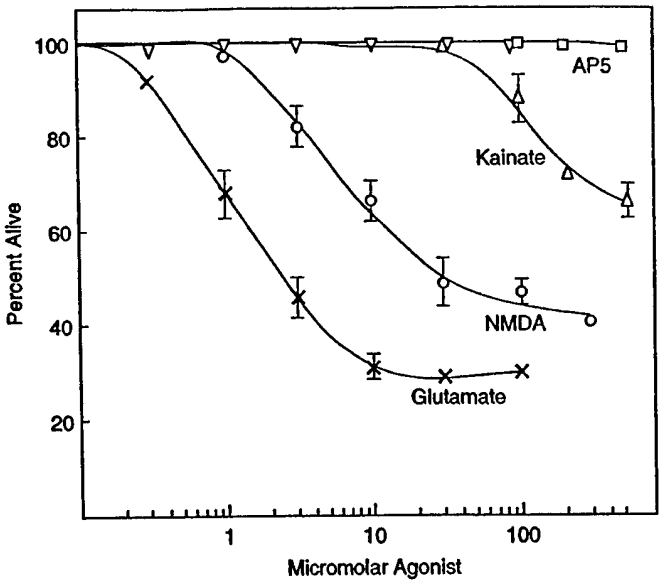

Figure 1. Ionotropic glutamate receptor-mediated toxicity. After 8 d in culture, E14 cortical neurons were exposed to the indicated reagents for 10 min, and cell viability was measured 24 hr later by the MTT assay, as described in Materials and Methods. The results were confirmed by visual (trypan blue exclusion) assays and are the mean of triplicate determinations ± SEM. x, Glutamate; ○, NMDA; △, kainate; ▽, glutamate plus 100 μM AP-5; □, AMPA.

Table 1. Toxicity of 10 μM glutamate

	EC_{50}		
Reagent	Day 8	Day 9	Day 10
GYKI-52466	>100 μM	>100 μM	>100 μM
AMOA	>300 μM	>300 μM	>300 μM
CNQX	>1000 μM	>1000 μM	>1000 μM
MK-801	500 nM	450 nM	450 nM
AP5	55 μM	50 μM	50 μM
DCQX	100 μM	100 μM	100 μM

Cells were exposed to varying concentrations of the reagents for 30 min before, during, and after exposure to 10 μM glutamate for 10 min. Cell death was determined 24 hr later by the MTT assay, and the concentrations that protected 50% of the cells from 10 μM glutamate toxicity are presented. The experiments were repeated at least three times with similar results. The > means that there is no effect at this concentration, the highest tested.

RESULTS

Cortical neuron cell death can be initiated by a purely NMDA receptor-dependent mechanism

As outlined above, there is some evidence for a non-ionotropic glutamate receptor component of the excitotoxicity cascade, but there have been only limited attempts to isolate and study this event. To do so, a number of criteria should be met. These include reproducibility, a pure nerve cell population to avoid confounding interactions with glia, a quantitative cytotoxic assay, and a system in which the process is initiated by the activation of a single class of ionotropic receptors, ideally NMDA receptors. By combining and modifying a number of published procedures (Rose et al., 1993) (also see, for example, Dugan et al., 1995), we devised a cell culture system that meets these criteria. Briefly, E14 mouse cortical neurons are dissociated and plated into 96-well microtiter plates in Neurobasal medium containing B27 supplements (Brewer et al., 1993) and fetal calf serum. Then 2 d later the medium is replaced with serum-free B27-supplemented medium alone containing cytosine arabinoside. The experiments are done between 7 and 14 d after plating, and cell viability usually is determined by the reduction of MTT (Liu et al., 1997) 24 hr after a 10 min exposure to glutamate. After 8 d in culture the cells are killed by glutamate with an EC_{50} of ~2 μM and by NMDA with an EC_{50} of 20 μM. AMPA and kainate are not toxic to these cells unless concentrations in excess of 100 μM are used (Fig. 1). The toxicity of 10 μM glutamate is blocked completely by the NMDA receptor antagonists AP-5, DCQX, and MK-801, but not by the kainate/AMPA antagonists CNQX, GYKI-52466, or AMOA (Table 1). These data show that the cytotoxic cascade in this culture system is initiated exclusively by the activation of NMDA receptors, therefore meeting the criteria for excitotoxicity as initially defined by Olney (1986).

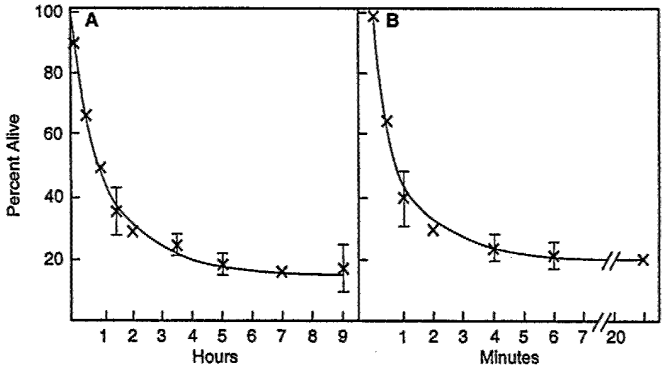

Figure 2. Temporal requirements for glutamate excitotoxicity. *A*, Cells 9 d in culture were exposed to 10 μM glutamate for 10 min, followed by a 3 hr MTT assay for viability at various times after glutamate exposure. For example, at 0 hr the cells were exposed to glutamate and assayed immediately for viability in the 3 hr MTT assay; the 5 hr point is a 5 hr incubation after glutamate, followed by a 3 hr MTT assay. *B*, Cells were exposed to 10 μM glutamate for 0–20 min, followed by the MTT viability assay 24 hr later. At the 30 sec time point ~35% of the cells died during the next 24 hr. The results are the mean of triplicate determinations ± SEM.

Cell death is initiated rapidly

To determine how rapidly cells die under the experimental conditions outlined above, we exposed cultures to 10 μM glutamate for 10 min, followed by a 3 hr MTT viability assay at various times after glutamate exposure. The results were confirmed by visual assays, including propidium iodide exclusion. Figure 2*A* shows that most of the cell death is quite rapid, with maximal levels at ~4 hr postglutamate exposure. The duration of exposure to 10 μM glutamate that is required to elicit maximum cell death is also short. When cells are exposed to 10 μM glutamate for various lengths of time, followed by a viability assay 24 hr later, cell death is significant after 1 min and maximum with a 3–4 min exposure (Fig. 2*B*). All cell death can be prevented by the inclusion of 100 μM AP-5 in the glutamate incubation medium. Therefore, there is a very efficient coupling between NMDA receptor activation and the initiation of the cell death pathways.

Cell death can be divided into three components

Although the initiation of cell death is totally dependent on the activation of NMDA receptors, it is possible that other forms of cell death are hidden within the ionotropic receptor-initiated process. To isolate a possible NMDA receptor-independent component, we exposed cells to 10 μM glutamate for 10 min and then cultured them continuously in the presence or absence of AP-5, a potent NMDA antagonist that completely blocks glutamate toxicity in these cultures (Table 1). Figure 3*A* shows that, at 8 d in culture, three components of the excitotoxicity cascade are revealed by this procedure. Approximately 80% of the cells are killed by a 10 min exposure to 10 μM glutamate (*arrow A*), and none are killed when AP-5 is present with glutamate. However, if AP-5 is added immediately after the exposure to glutamate, ~30% of the cells are rescued from cell death (*arrow B*). It follows that the 30% of the cells that are rescued by AP-5 require NMDA receptor activation after glutamate exposure, whereas the remaining 50% (*arrow C*) must be killed either by the initial exposure to glutamate via the activation of NMDA receptors or by a downstream mechanism that is independent of the NMDA receptor. If AP-5 is present during the exposure to glutamate and then removed from the cultures, there is still no cell death, for under these conditions glutamate cannot activate receptors and initiate the cascade. These observations are consistent with previous observations showing that a significant fraction of cells destined to die after glutamate exposure can be rescued by NMDA antagonists applied after the initial glutamate exposure (Rothman et al., 1987; Hartley and Choi, 1989; Manev et al., 1989). Approximately 20% of the cells never die under these conditions; the reason for this is unknown.

The interpretation of these data, and the basis for the following experiments, is that the activation of NMDA receptors during the 10 min exposure to 10 μM glutamate initiates the death of a population of cells, which is represented within the "A" and "C" components. This event triggers two additional responses caused by the initial lysis of cells and the accumulation of glutamate in the culture medium. One is the subsequent activation of NMDA receptors on additional cells, resulting in more receptor-dependent cell death (population B); the other possible outcome is the death of a population of cells that do not have functional NMDA receptors (a subset of population C). These alternatives are shown schematically in Figure 3*B*, in which the circles on the left represent cells directly killed during the 10 min glutamate exposure and the circles on the right are cells killed after glutamate exposure via NMDA (*circled N*) and NMDA receptor-independent (*open circles*) mechanisms. The experiments below define the cell death pathway by which this latter population is killed.

Glutamate receptor expression changes with length of time in culture

It has been observed repeatedly that the efficiency of excitotoxic cell death is dependent on the length of time the cells have been maintained in culture (see, for example, Dugan et al., 1995). This is presumably attributable to the time required for the cells to express functional ionotropic receptors. To assay the distribution of NMDA receptor versus non-NMDA receptor-mediated killing as a function of time in culture, we repeated the experiment described in Figure 3 on days 7–11 of cell culture. The fraction of the total nerve cell culture that is killed by a 10 min exposure to 10 μM glutamate increases from 40% at day 7 to ~80% on days 10 and 11 (Fig. 4). In contrast, ~60% of the cells that die are rescued by the postglutamate addition of AP-5 at day 7. This decreases to 20% between days 10 and 11.

The observation that the total number of cells killed increases with culture age suggests that either the level of NMDA receptor expression increases or its coupling to relevant second message systems is dependent on the amount of time the neurons are in culture. Because one NMDA receptor subunit, NR1, is common to most NMDA ionotropic channels (for review, see Akazawa et al., 1994), the expression of this subunit was followed by Western blotting as a function of time in culture. Figure 5*A* shows that the expression of the NR1 receptor dramatically increases between days 3 and 10 in culture, suggesting that NMDA receptor availability may be limited in the NMDA receptor-mediated killing. Concomitant with culture age is an increase in neurite density (data not shown). Actin is a major component of neurites, and the amount of actin in neuronal cultures correlates with neurite density. Figure 5*A* shows that there is an increase in actin accumulation closely paralleling that of NR1, suggesting that most of the NR1 may be associated with neurites.

In addition to ionotropic receptors, glutamate activates metabotropic receptors (mGluRs). mGluR activation has been associated with a variety of physiological processes, including protection from oxidative glutamate toxicity (Sagara and Schu-

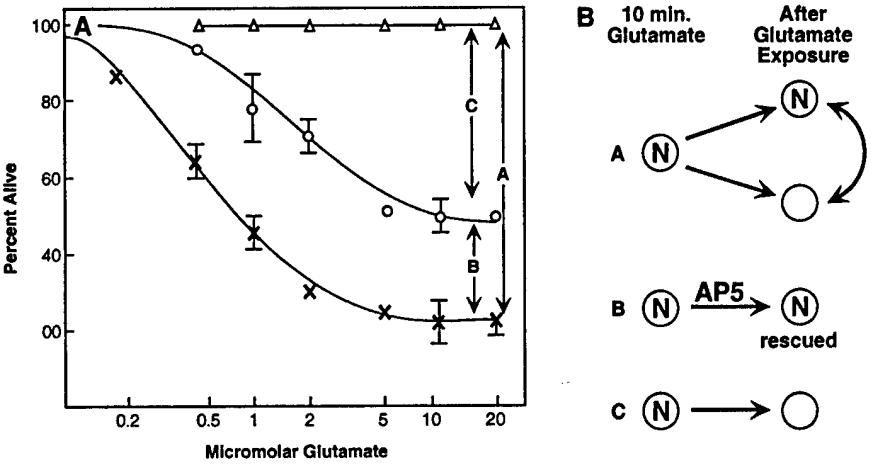

Figure 3. A portion of excitotoxic cell death is non-NMDA receptor-mediated. *A*, After 8 d in culture, E14 cortical neurons were exposed for 10 min to the indicated concentrations of glutamate in the presence or absence of AP-5 and then incubated for 24 hr in the presence or absence of AP-5, at which time cell viability was monitored by the MTT assay. x, Glutamate alone; △, glutamate plus 100 μM AP-5 during and after the 10 min glutamate exposure; ○, glutamate plus 100 μM AP-5 added immediately after glutamate exposure. *A* indicates total cell death in the system. *B* indicates the fraction of cells that die after glutamate exposure by a NMDA receptor-mediated process. *C* indicates the fraction of cells that die by virtue of the initial NMDA activation of the cell death pathway plus those that die independently of the NMDA receptor after the initial exposure to glutamate. *B*, Schematic representation of alternative cell death pathways identified above. *Open circles* represent cells lacking NMDA receptors and *circles* enclosing an *N* represent cells with functional NMDA receptors. The *two-headed arrow* in *A* indicates that there may be a reciprocal interaction leading to cell death between cells with and without NMDA receptors.

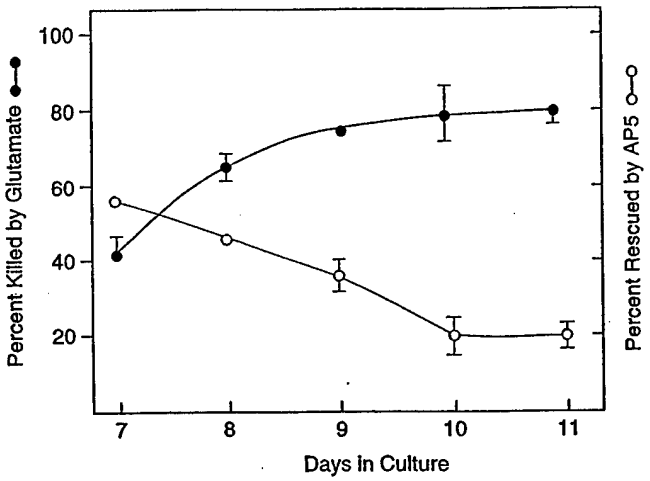

Figure 4. Changes in cell death mechanism as a function of time in culture. E14 cortical cultures were monitored for glutamate-induced cell death exactly as described in Figure 3 but as a function of time in culture. The endpoint that is plotted is the plateau of killing by 10 μM glutamate (see Fig. 3). ●, Percentage of the initial cell population killed by glutamate (10 min exposure); ○, percentage of total late cell death in the culture rescued by AP-5 (see Fig. 3*B*). The data are the mean ± SEM of three or four experiments.

bert, 1998). Therefore, the expression of mGluRs 1, 3 and 4, and 5 was monitored by Western blotting in the same lysates as NR1 and actin. Figure 5 shows that all of these receptors are expressed in the cortical cultures but that their expression patterns vary. The expression of mGluRs 1 and 5 increases with time in culture until day 7, after which their expression declines. In contrast, the expression of mGluRs 2 and/or 3 increases with culture age in a manner similar to that of NR1 and actin.

Oxidative glutamate toxicity is a component of excitotoxicity

Oxidative glutamate toxicity is a well studied programmed cell death pathway that is independent of ionotropic glutamate receptors (Murphy et al., 1989; Maher and Davis, 1996; Li et al., 1997a,b; Tan et al., 1998a,b). If oxidative glutamate toxicity is a component of excitotoxicity, then it should be inhibited by reagents that selectively block oxidative glutamate toxicity, but not by AP-5. If a compound blocks the NMDA-mediated component in addition to oxidative glutamate toxicity, then the whole cascade would be inhibited because its initiation is dependent on NMDA receptor activation. Therefore, to determine whether oxidative glutamate toxicity is involved in the excitotoxicity pathway, a variety of components that inhibit oxidative glutamate toxicity but do not block excitotoxicity were screened for their ability to block the C fraction of the excitotoxicity cascade (see Fig. 3).

A defining characteristic of oxidative glutamate toxicity is that it is strongly inhibited by many antioxidants, including vitamin E (Murphy et al., 1989). To determine whether part of the C component shares this trait with oxidative glutamate toxicity, we preincubated 8-d-old cultures of cortical cells for 30 min with 100 μM α-tocopherol, followed by glutamate exposure and a 24 hr incubation with α-tocopherol ± AP-5. Figure 6*A* shows that part of the C phase of cell death is blocked by α-tocopherol, whereas the viability of the cells exposed to glutamate in the absence of AP-5 is increased by the same amount. This increase in viability is expected in the absence of AP-5, because this condition contains both the NMDA receptor-independent and NMDA receptor-mediated components of glutamate toxicity. Because one-half of the cells survive at day 8 in the presence of α-tocopherol and because α-tocopherol has no effect on excitotoxicity at days 10 and 11 (data not shown), α-tocopherol must not block the NMDA receptor-mediated excitotoxicity component. Although these results are consistent with oxidative glutamate toxicity being a component of the excitotoxicity cascade, a number of other reagents known to inhibit oxidative glutamate toxicity were examined also. These include the group I metabotropic glutamate receptor (mGluR1) agonists and a caspase 1 inhibitor that has been shown previously to block oxidative glutamate toxicity (Tan et al., 1998a,b).

The activation of group I mGluRs protects cells from oxidative glutamate toxicity via the activation of the inositol triphosphate pathway (Sagara and Schubert, 1998). If we use the same logic applied to the experiments with vitamin E, if oxidative glutamate toxicity is a component of excitotoxicity, then mGluR1 agonists should inhibit part of component C of the cascade. Figure 6, *B* and *C*, shows that two mGluR agonists, (*R,S*)-3,5-dihydroxyphenylglycine (DHPG) and *trans*-1-amino-1*S*,3*R*-cyclopentane dicarboxylic acid (ACPD), both protect from excitotoxic-initiated glutamate damage in 8 d cultures. It also has been shown elsewhere that ACPD has a partial protective effect on NMDA-

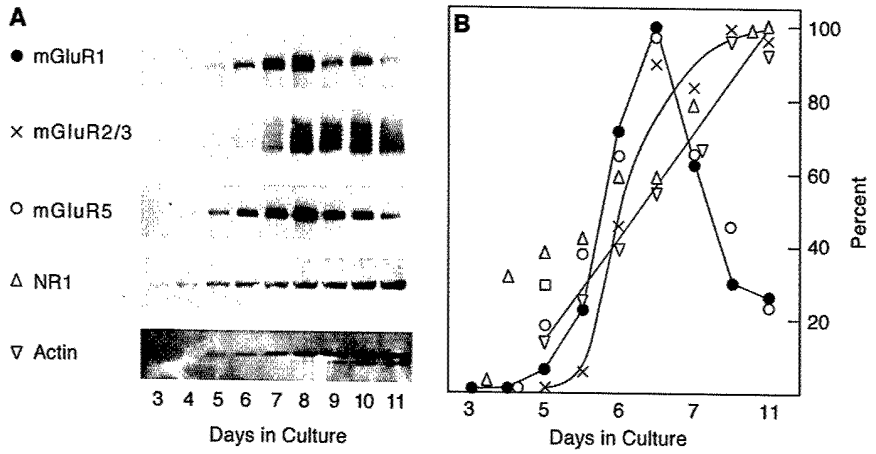

Figure 5. Expression of glutamate receptors as a function of time in culture. Cell lysates were made from E14 cortical neurons cultured for 3–11 d. Then the lysates were run on SDS-acrylamide gels and immunoblotted with the indicated anti-receptor antibodies. The same fraction of each culture dish was loaded per lane; the amount of protein per culture increased only ~20% from day 7 to 11. Quantitation was accomplished by scanning the negatives. *A*, *Top*, mGluR1; mGluR2/3; mGluR5; NMDA NR1; *A*, *Bottom*, Actin. The experiments were repeated at least three times with similar results. *B*, Quantitation: ●, mGluR1; x, mGluR2/3; ○, mGluR5; △, NR1; ▽, actin, shown as a percentage of maximal expression.

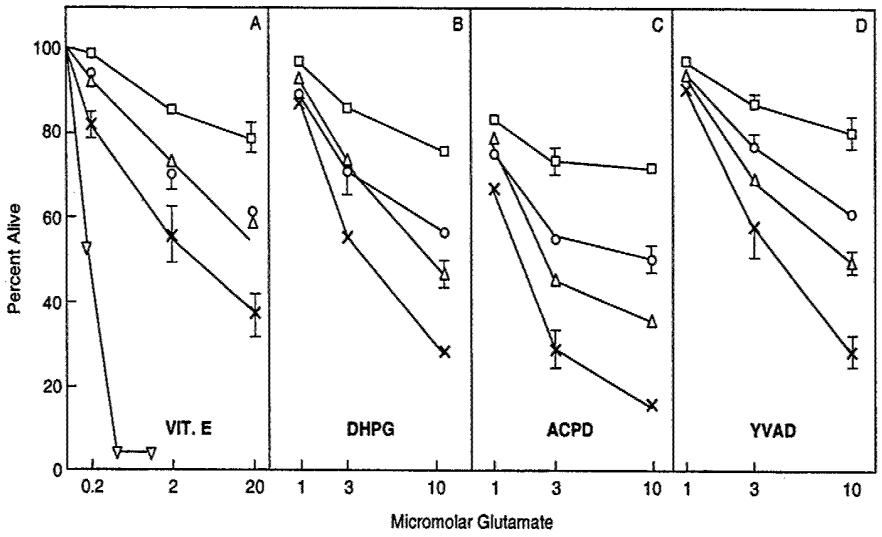

Figure 6. Conditions that block oxidative glutamate toxicity partially protect from excitotoxic-initiated damage. *A*, α-Tocopherol protects from cell death. Cells cultured for 8 d were pretreated for 30 min with 100 μM α-tocopherol (natural), exposed to 10 μM glutamate for 10 min, and then returned to the original medium ± AP-5, ± α-tocopherol. x, Glutamate alone; ○, glutamate plus α-tocopherol; △, glutamate plus 100 μM AP-5 after glutamate exposure; □, glutamate plus α-tocopherol plus AP-5 after glutamate; ▽, cell viability at 24 hr after continuous exposure to glutamate plus 100 μM AP-5, 100 μM GYKI-25466, and 500 μM CNQX. Multiply glutamate concentration by 1000 (e.g., complete killing at 500 μM glutamate). *B*, *C*, Group I mGluR activation is protective. Cells were pretreated for 30 min with 100 μM mGluR agonists DHPG (*B*) or ACPD (*C*), followed by a 10 min exposure to the indicated concentrations of glutamate. Then the original culture medium was returned to the cells along with the mGluR reagents and, in some cases, 100 μM AP-5 to block downstream NMDA receptor activation. Cell viability was determined 24 hr later by the MTT assay. x, Glutamate alone; △, glutamate plus agonist; ○, glutamate plus AP-5 after glutamate exposure; □, glutamate plus AP-5 after the added agonist. *D*, A caspase inhibitor Ac-YVAD-cmk protects cells. Cells were exposed to 30 μM Ac-YVAD-cmk for 30 min before exposure to 10 μM glutamate. In some cases 100 μM AP-5 was present throughout. x, Glutamate alone; △, glutamate plus caspase inhibitor; ○, glutamate plus AP-5; □, glutamate plus AP-5 after the added caspase inhibitor.

mediated excitotoxicity (Koh et al., 1991). Another agent that protects cortical neurons from oxidative glutamate toxicity is Ac-YVAD-cmk, a potent caspase inhibitor (Tan et al., 1998a,b). Figure 6*D* shows that this inhibitor protects cells in the presence of AP-5 by ~20%. These data again substantiate the involvement of oxidative glutamate toxicity as the cause of between 20 and 30% of the cell death in the excitotoxicity cascade.

The vitamin E, the mGluR agonist, and the caspase inhibitor data show that under certain conditions excitotoxicity can be divided into three components, one of which has the characteristics of oxidative glutamate toxicity. In older cultures (10–11 d) only 20% of the cell death is blocked by the late application of AP-5, and no cell death is blocked by the oxidative glutamate toxicity antagonists described above (see Fig. 4; data not shown). These data show that the oxidative glutamate toxicity component of excitotoxicity is transient in these cultures and strongly support the argument that vitamin E, DHPG, ACPD, and Ac-YVAD-cmk do not inhibit the NMDA receptor-mediated response. The transient nature of the oxidative glutamate toxicity response may be attributable to the fact that the NMDA receptor-mediated response is more efficient in older cultures because of higher receptor density (see Fig. 5) or the loss of cells that do not express NMDA receptors from the older cultures. This also would result in a larger fraction of the cells being killed by initial glutamate exposure.

Soluble glutamate mediates late cell death

Because it is likely that the late cell death outlined above is attributable to glutamate, the amount of free glutamate in the culture medium was assayed as a function of time after the addition of 10 μM glutamate for 10 min. The amount of free glutamate increased from undetectable levels (<10 μM) to ~300 μM over a period of 9 hr (Fig. 7*E*). Glutamate (300 μM) is sufficient to inhibit extracellular cystine uptake completely, deplete intracellular GSH in clonal nerve cells (Sagara and Schubert, 1998), and kill >50% of the cells in this culture system via oxidative glutamate toxicity, as determined by the long-term exposure to glutamate in the presence of high concentrations of NMDA, AMPA, and kainate antagonists (see Fig. 6*A*, *inverted triangles*). These data clearly show that extracellular glutamate in these cultures can reach concentrations sufficient to cause damage via the oxidative glutamate toxicity pathway. The inclusion of 100

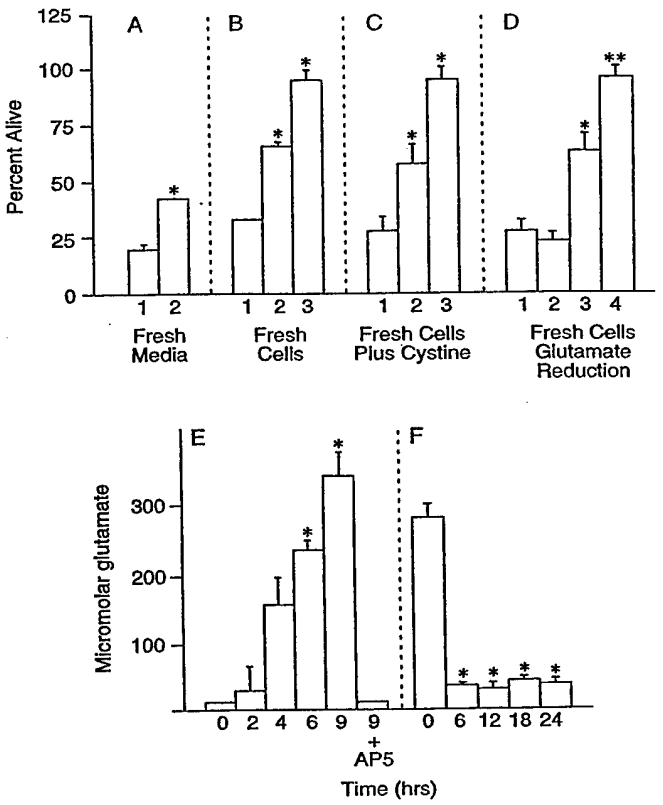

Figure 7. Toxicity is transferred by conditioned medium. Cell viability was measured after 24 hr in all cultures. *A*, Cells cultured for 8 d were exposed to 10 μM glutamate for 10 min, washed once, and returned to their original growth medium. After 11 hr either the cells were given fresh culture medium (*2*) or the medium was left undisturbed (*1*). *B*, In another experiment the medium was transferred to new cells of identical age in the absence (*1*) or presence (*2*) of 100 μM AP-5 or in the presence of AP-5 and 30 μM Ac-YVAD-cmk (*3*). *C*, Cells were exposed to growth-conditioned medium alone (*1*) or in the presence of 2 mM cystine (*2*) or in the presence of 2 mM cystine and 100 μM AP-5 (*3*). Because of the relative insolubility of cystine below pH 8, the experiments with cystine and all controls were performed at pH 8 by the reduction of incubator CO_2. *D*, After 11 hr of glutamate exposure, growth-conditioned media were in some cases (*2*, *4*) pretreated for 2 hr with GPT to reduce glutamate and then were transferred to fresh cells. *1*, Growth-conditioned medium alone; *2*, medium treated with GPT; *3*, untreated medium plus AP-5; *4*, glutamate-depleted medium plus AP-5. ***Bar 4* is significantly different from *bar 3*, $p < 0.01$; $n = 3$. *E*, Concentration of glutamate in the growth-conditioned medium as a function of time after the addition of 10 μM glutamate to cultures. In one set of cultures 100 μM AP-5 was added before the addition of 10 μM glutamate (9 hr plus AP-5). *F*, Reduction of glutamate in the medium by GPT. GPT and cofactors were added to the growth-conditioned medium before application to the cells initially for 2 hr; then GPT was added repeatedly every 6 hr during the experiment to keep extracellular glutamate below 50 μM. *Significantly different from control (conditioned medium alone), $p < 0.05$; $n = 3$.

μM AP-5 during glutamate exposure completely blocked extracellular glutamate accumulation (Fig. 7*E*). The glutamate concentrations are higher than previously reported in some cell culture systems (Strijbos et al., 1996), most probably because of the absence of astrocytes to remove free glutamate, but are very similar to those found in the culture media of lysed neurons (Newcomb et al., 1997).

The above data show that there can be an ionotropic receptor-independent component of excitotoxicity, that the latter can be accounted for by the oxidative glutamate pathway, and that there is a high concentration of glutamate in media from lysed cells. If these conclusions are valid, then four additional criteria should be met. (1) Cells previously exposed to glutamate should be protected by replacing their conditioned medium with fresh medium. (2) It should be possible to transfer the late toxicity via the growth-conditioned medium. (3) Elevated exogenous cystine should reverse the inhibition by glutamate and protect cells. (4) The removal of glutamate from the conditioned medium should block downstream oxidative toxicity. Figure 7*A* shows that conditioned medium replacement with fresh medium after 11 hr reduces subsequent cell death by ~20% (Fig. 7*A*, *bar 2*). Figure 7*B* shows that ~30% of the cell death caused by the transfer of 11 hr conditioned medium from cells treated for 10 min with 10 μM glutamate to fresh cells of the same age is blocked by AP-5 (Fig. 7*B*, *bar 2*) and that an additional 30% is blocked by the caspase inhibitor YVAD (Fig. 7*B*, *bar 3*).

Because the x_c^- antiporter is inhibited by glutamate in a competitive manner (Sato et al., 1999), it should be possible to reverse the effect of exogenous glutamate with cystine. By increasing the concentration of cystine in the culture medium (normally 260 μM) ~10-fold, we have shown that cells were protected from conditioned medium by ~30% (Fig. 7*C*, *bar 2*), with almost complete protection by a combination of cystine and AP-5 (Fig. 7*C*, *bar 3*). These data, in conjunction with those presented in Figure 6, strongly suggest that the oxidative glutamate toxicity pathway can kill some of the cells in excitotoxic pathways.

Finally, exogenous glutamate was reduced in the growth-conditioned medium by treatment of the medium with 100 μg/ml GPT, 100 μM pyridoxal-L-phosphate, and 10 mM pyruvate (Matthews et al., 2000). Figure 7*F* shows that GPT treatment reduced glutamate from 300 to ~40 μM. In the absence of AP-5 there should be a level of cell death caused by reduced glutamate medium similar to conditioned medium alone, because the residual glutamate is sufficient to activate NMDA receptors and all downstream cell death pathways (Fig. 7*D*, *bars 1*, *2*). In the presence of AP-5, which blocks all NMDA receptor-mediated events, oxidative glutamate toxicity still should occur in the high glutamate medium. However, when extracellular glutamate is reduced to a level at which it can initiate NMDA receptor-mediated toxicity but not oxidative glutamate toxicity, all of the toxicity should be eliminated in the presence of AP-5. The data in Figure 7*D* again support a role for oxidative glutamate toxicity, for AP-5 only partially inhibits the cell death in high glutamate medium (Fig. 7*D*, *bar 3*), whereas there is 100% survival in glutamate-depleted medium plus AP-5 (Fig. 7*D*, *bar 4*).

DISCUSSION

The above data show that the excitotoxicity cascade can be divided experimentally into three discrete components, two requiring the activation of NMDA receptors. The initiation of the cell death pathway requires NMDA receptor activation, and a second NMDA receptor-dependent phase takes place after a brief exposure to low concentrations of glutamate. In contrast, a distinct form of cell death can occur after glutamate exposure that is independent of ionotropic glutamate receptors. This pathway, which constitutes 20–30% of the total cell death in 8–9 d cultures, has characteristics of oxidative glutamate toxicity, for it is inhibited specifically by vitamin E, by group I metabotropic receptor agonists, by a caspase inhibitor, by elevated extracellular cystine, and by the removal of extracellular glutamate. These data explain earlier observations showing that there is significant cell death in excitatory amino acid toxicity, ischemia, and CNS trauma, which is

independent of ionotropic glutamate receptors (Meldrum and Garthwaite, 1990) (also see, for example, Choi, 1992).

In cultures of hippocampal neurons, approximately one-half of the cells can be rescued by applying NMDA antagonists after glutamate exposure (Rothman et al., 1987; Hartley and Choi, 1989; Manev et al., 1989). These data and those presented above show that there is an initial population of cells that is killed by glutamate exposure directly and another population that dies later because of the activation of NMDA receptors. The late receptor-mediated cell death could be attributable to either the requirement for a subset of NMDA receptors that respond to the higher concentrations of extracellular glutamate derived from cell lysis or have a requirement for more prolonged exposure to cell-derived glutamate. In our experiments, of the cells that cannot be rescued by the late application of AP-5, approximately one-half die by a process with the characteristics of oxidative glutamate toxicity. The other one-half die because of the initial exposure to glutamate and require NMDA receptor activation.

Previous studies have shown that the activation of different classes of ionotropic glutamate receptors is dependent on glutamate concentration, exposure time, and probably on the cell population. For example, unlike for NMDA, a brief exposure of cortical cells to AMPA and kainate produces little cell death, but exposure of the cells to these receptor agonists for hours produces extensive cell death (Choi et al., 1989; Frandsen et al., 1989). This may be because most AMPA/kainate receptors are relatively impermeable to Ca^{2+}, requiring the activation of voltage-dependent Ca^{2+} channels for toxicity. In addition to exposure duration, AMPA/kainate receptor-mediated cell death is much slower, requiring many hours for cell lysis to occur (Choi, 1992; Carriedo et al., 1998), and these later forms of cell death have some characteristics of apoptosis (Choi and Rothman, 1990; Kure et al., 1991). However, because AMPA/kainate receptor antagonists have no effect in this culture system (see Table 1), even when added after glutamate exposure (data not shown), it is unlikely that these receptors play a role in the cell death that occurs after transient glutamate exposure. However, consistent with most of the published literature is the observation that some downstream cell death occurs by a mechanism that has many characteristics of programmed cell death, such as caspase activation (Tan et al., 1998a,b). This cell death pathway is oxidative glutamate toxicity.

Oxidative glutamate toxicity requires higher concentrations of glutamate than are necessary for NMDA receptor activation (Murphy et al., 1989). Figure 7 shows that concentrations of extracellular glutamate in the 200–300 μM range are present in cultured cells after initial excitotoxic cell lysis; these concentrations are sufficient to cause oxidative glutamate toxicity (see Fig. 6*A*). Similar concentrations of extracellular glutamate have been reported in culture media of lysed neurons (Newcomb et al., 1997) and in CNS trauma models (McAdoo et al., 1999). Because the culture medium contains 2 mM glutamine and nerve cells possess a very active enzyme, glutaminase, which converts glutamine to glutamate, initial nerve cell lysis releases this enzyme that, in the presence of abundant substrate, leads to an accumulation of glutamate in the culture medium (Newcomb et al., 1997). The brain also contains concentrations of glutamine between 2 and 4 mM, with 0.5 mM found in CSF (Matsumoto et al., 1996). Because this culture system lacks glial cells and many of the nerve cells are damaged rapidly, there is no effective way of removing glutamate. During ischemia, trauma, and other pro-oxidant conditions there is also likely to be a loss of high-affinity glutamate transporter function because these molecules are exquisitely sensitive to biological oxidants (for review, see Trotti et al., 1998).

In oxidative glutamate toxicity, glutamate blocks the cystine/glutamate exchange system x_c^-, resulting in glutathione depletion and cell death (Murphy et al., 1989). The molecular basis of x_c^- function has been described recently (Sato et al., 1999). The exchange systems consist of two proteins, the heavy chain of 4F2 (4F2hc) that is involved in several amino acid transport systems and a 502 amino acid protein called XCT. Both XCT and 4F2hc are highly expressed in the brain (Kanai et al., 1998; Sato et al., 1999). Because the cells of the CNS contain sequestered concentrations of free glutamate in the millimolar range (Coyle et al., 1981), as well as the ability to convert glutamine to glutamate, it is probable that any cellular dysfunction, such as loss of energy metabolism or cell lysis, would create local concentrations of glutamate sufficient to inhibit glutamate uptake and subsequent glutathione synthesis in nearby cells. The EC_{50} glutamate concentration for inhibiting cystine uptake is <100 μM (Sagara and Schubert, 1998), and ~200 μM extracellular glutamate kills 50% of the cortical neurons used in the above experiments via oxidative glutamate toxicity (see Fig. 6*A*). This sequence of events could lead to cell injury or death in an autocatalytic manner, resulting in a gradient of injury radiating from the site of the initial event. In addition, oxidative glutamate toxicity can generate even greater damage than excitotoxicity, because neurons lacking ionotropic glutamate receptors are killed also. It is therefore of importance to understand how x_c^- is regulated in the brain as well as how oxidative glutamate toxicity kills neurons.

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Regulation of Antioxidant Metabolism by Translation Initiation Factor 2α

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Abstract. Oxidative stress and highly specific decreases in glutathione (GSH) are associated with nerve cell death in Parkinson's disease. Using an experimental nerve cell model for oxidative stress and an expression cloning strategy, a gene involved in oxidative stress–induced programmed cell death was identified which both mediates the cell death program and regulates GSH levels. Two stress-resistant clones were isolated which contain antisense gene fragments of the translation initiation factor (eIF)2α and express a low amount of eIF2α. Sensitivity is restored when the clones are transfected with full-length eIF2α; transfection of wild-type cells with the truncated eIF2α gene confers resistance. The phosphorylation of eIF2α also results in resistance to oxidative stress. In wild-type cells, oxidative stress results in rapid GSH depletion, a large increase in peroxide levels, and an influx of Ca^{2+}. In contrast, the resistant clones maintain high GSH levels and show no elevation in peroxides or Ca^{2+} when stressed, and the GSH synthetic enzyme γ-glutamyl cysteine synthetase (γGCS) is elevated. The change in γGCS is regulated by a translational mechanism. Therefore, eIF2α is a critical regulatory factor in the response of nerve cells to oxidative stress and in the control of the major intracellular antioxidant, GSH, and may play a central role in the many neurodegenerative diseases associated with oxidative stress.

Key words: oxidative stress • glutathione • eIF2α • resistance • glutamate

Introduction

Although programmed cell death (PCD)[1] is a widely used mechanism for sculpturing the developing nervous system, its inappropriate activation leads to premature nerve cell death in neuropathological disorders such as Alzheimer's disease (AD) (Yankner, 1996) and Parkinson's disease (PD) (Mochizuki et al., 1996). Nerve cell death in both PD and AD are thought to be linked to oxidative stress, as antioxidant systems are upregulated and there is extensive evidence for excessive lipid and protein peroxidation (Jenner and Olanow, 1996; Simonian and Coyle, 1996). Associated with oxidative stress is an early and highly specific decrease in the glutathione (GSH) content of the substantia nigra of PD patients (Perry et al., 1982; Sofic et al., 1992; Sian et al., 1994) which may precede the death of dopaminergic neurons (Dexter et al., 1994). In addition, the inhibition of γ-glutamyl cysteine synthetase (γGCS), the rate-limiting step in GSH synthesis, results in the selective degeneration of dopaminergic neurons (Jenner and Olanow, 1996) and also potentiates the toxicity of 6-hydroxydopamine, MPTP and MPP^+. These data suggest that GSH and oxidative stress play pivotal roles in the pathogenesis of AD and PD.

There are several ways in which the concentration of intracellular GSH and the oxidative burden of cells can be regulated. One of these is through extracellular glutamate. Although glutamate is generally thought of as both a neurotransmitter and an excitotoxin, extracellular glutamate can also kill neurons through a non–receptor-mediated pathway which involves the glutamate-cystine antiporter, system Xc^- (Bannai and Kitamura, 1980; Murphy et al., 1989; Sato et al., 1999). Under normal circumstances, the concentration of extracellular cystine is high relative to intracellular cystine, and cystine is imported via the Xc^- antiporter in exchange for intracellular glutamate. Cystine is ultimately converted to cysteine and used for protein synthesis and to make the antioxidant GSH. However, when there is a high concentration of extracellular glutamate, the exchange of glutamate for cystine is inhibited, and the cell becomes depleted of cysteine and GSH, resulting in severe oxidative stress. The cell eventually dies via a series of events which include the depletion of GSH, a requirement for macromolecular synthesis and caspase activity, lipoxygenase activation, soluble guanylate cyclase activation, reactive oxygen species (ROS) accumulation, and finally Ca^{2+} influx (Murphy et al., 1989; Li et al., 1997a,b; Tan et al., 1998a,b). PCD caused by oxidative glutamate

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[1]*Abbreviations used in this paper:* AD, Alzheimer's disease; DCF, dichlorofluorescein; eIF, translation initiation factor; GSH, glutathione; γGCS, gamma-glutamyl cysteine synthetase; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PCD, programmed cell death; PD, Parkinson's disease; ROS, reactive oxygen species.

toxicity has characteristics of both apoptosis and necrosis (Tan et al., 1998a) and has been well studied in primary neuronal cell cultures (Murphy and Baraban, 1990; Oka et al., 1993), neuronal cell lines (Miyamoto et al., 1989; Murphy et al., 1989), tissue slices (Vornov and Coyle, 1991), and in the immortalized mouse hippocampal cell line, HT22 (Li et al., 1997a,b; Tan et al., 1998a,b). HT22 cells lack ionotropic glutamate receptors but die within 24 h after exposure to 1–5 mM glutamate. Although the biochemical events have been well studied, little has been done to identify the transcriptional/translational changes that contribute to the glutamate-induced pathway of PCD. Changes in gene expression clearly play a role in the cell death cascade since macromolecular synthesis is required early in the death pathway (Tan et al., 1998a,b). Through the use of a genetic screen, we identified the α subunit of the translation initiation factor 2 (eIF2α) as a gene whose expression is involved in oxidative stress–induced cell death and the regulation of intracellular GSH. eIF2 is a trimeric complex involved in the initiation of translation (Hershey, 1991; Pain, 1996). The complex is made up of three subunits designated alpha, beta, and gamma, and behaves in a manner analogous to the trimeric G-coupled proteins. The α subunit dictates whether protein synthesis will or will not take place and is often referred to as the control point for protein synthesis. The eIF2 complex brings the 40S ribosomal subunit together with the initiating $tRNA_{met}$ when eIF2α is bound to GTP. Upon hydrolysis of GTP to GDP, the complex is no longer active and protein synthesis is not initiated. GDP/GTP exchange takes place readily with the assistance of a guanine nucleotide exchange factor, eIF2B. However, when the α subunit of eIF2 is phosphorylated on serine 51, a change in the conformation enables it to bind and sequester eIF2B, thus inhibiting GDP/GTP exchange and protein synthesis. eIF2α phosphorylation takes place during ischemia (DeGracia et al., 1997; Burda et al., 1998), apoptosis (Srivastava et al., 1998; Satoh et al., 1999), viral infection (Samuel, 1993; Wek, 1994), and after Ca^{2+} influx (Prostko et al., 1995; Srivastava et al., 1995; Reilly et al., 1998). Therefore, eIF2α may have significant roles in the cell death process after oxidative stress that are separate from its known function as a regulator of protein synthesis. The experiments described below show that the downregulation or phosphorylation of eIF2α protects nerve cells from oxidative stress–induced cell death by inhibiting GSH depletion and the increase in both ROS and intracellular Ca^{2+} that are normally seen in cells exposed to oxidative stress. These data demonstrate a unique role of eIF2α in oxidative stress–induced programmed nerve cell death, acting as a translational switch which dictates whether a cell activates a survival response or follows a cell death pathway. eIF2α may therefore play a central role in neuropathologies involving nerve cell death which are associated with oxidative stress.

Materials and Methods

The following chemicals were purchased from Sigma-Aldrich: puromycin, TCA, formic acid, GSH, GSH reductase, triethanolamine, sulfosalicylic acid, NADPH, BSA, glutaraldehyde, and L-glutamic acid (glutamate). The fluorescent probes 2′,7′-dichlorofluorescein (DCF) diacetate and indoacetoxymethylester (Indo-1), pluronic F-127, and propidium iodide were obtained from Molecular Probes. The Coomassie Plus protein assay reagent and the SuperSignal substrate were both purchased from Pierce Chemical Co. Immobilon P was purchased from Millipore.

Infection with the Retroviral cDNA Library

HT22 cells were infected with the retroviral vector pcLXSN containing a cDNA library derived from the human embryonic lung cell line, MRC-5 (Somia et al., 1999). The library contained 2×10^6 cDNAs, and the HT22 cells were infected with $\sim 10^7$ virus particles. The cDNA library contains both sense and antisense sequences. The retrovirus stably integrates into the host cell's genomic DNA and expresses the cDNA inserted between its long terminal repeats. Clones containing genes that confer glutamate resistance were identified by selecting cells that survived in 10 mM glutamate. Genomic DNA from each clone was analyzed by PCR using primers that straddle the cDNA insert in the retroviral vector. The cDNA inserts were then subcloned and sequenced. Viral vectors were rescued from the clones by transfection with an ecotropic helper plasmid. These viral particles were collected from the media and used to infect the packaging cell line, PA317, which amplified the virus (Miller et al., 1993). The viral medium from the packaging cells was then used to infect wild-type HT22 cells in order to confirm that the cDNA was indeed able to make the HT22 cells resistant to glutamate.

Immunoblotting and Northern Blot Procedures

Cells were plated at 5×10^5 cells per 100-mm dish 12–16 h before use and lysed in sample buffer containing 3% SDS. Lysates were sonicated, protein concentrations were normalized using the Coomassie Plus protein assay reagent from Pierce Chemical Co., and 25 μg protein was loaded per lane on 12% Tris-glycine SDS-PAGE gels (Novex). Gels were transferred onto Immobilon P membrane (Millipore) and blocked with 5% milk in TBS for 1 h at room temperature. An antibody against eIF2α (Research Genetics) was shown previously to recognize only phosphorylated eIF2α. However, in our hands the antibody recognized both phosphorylated and unphosphorylated protein when the Western blots and lysates were dephosphorylated with a mixture of bovine and calf intestine alkaline phosphatase. Blots were also probed with antibodies against both phosphorylated and total mitogen-activated protein kinase to confirm that proteins were completely dephosphorylated after treatment with the phosphatases. Therefore, this anti-eIF2α antibody was used to determine the levels of total eIF2α in the HT22 cells and the resistant clones 8 and 15. The anti-eIF2α primary antibody was diluted into 5% BSA in TBS plus Tween 20 (TTBS) at 1:250 and placed on the blot overnight at 4°C. Blots were incubated with the secondary antibody, goat anti–rabbit IgG HRP conjugated (Bio-Rad Laboratories), for 1 h at room temperature at a dilution of 1:20,000 in 5% milk in TTBS. Blots were exposed to Eastman Kodak Co. X-OMAT Blue film for chemiluminescence using the SuperSignal substrate from Pierce Chemical Co.

Northern blots of the γGCS catalytic subunit were done as described in the original paper in which cDNA clones were isolated (Gipp et al., 1992). Northern blots were done using a probe consisting of the COOH-terminal 387 amino acids of the protein which detected a single band of ~3.7 kb.

Transfection of Full-Length eIF2α into Clones 8 and 15

The full-length cDNA for eIF2α was obtained from Dr. Miyamoto (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) and was cloned into the pCLBABEpuro retroviral vector, a modified version of the pBABEpuro vector (Morgenstern and Land, 1990). This vector was then used for transfection with Lipofectamine (GIBCO BRL).

Production of Retrovirus Expressing the Dominant Negative Mutants of eIF2a

The cDNA constructs for two mutants of eIF2α (S51A and S51D) were obtained from Dr. Kaufman (University of Michigan, Ann Arbor, MI) and subcloned into pCLBABEpuro. Retroviral vectors were made as described (Somia et al., 1999) with either pCLBABE-S51A, pCLBABE-S51D, or pCLBABEpuro alone. The viral vectors were used to infect HT22 cells, and infected cells were selected in 4 μg/ml puromycin (Sigma-Aldrich). The puromycin-resistant cells were tested for glutamate resistance by the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) cell death assay (Tan et al., 1998b).

Translation and Degradation Assays

For translation assays, cells were labeled in 60-mm dishes with 500,000 cpm of [^{3}H]leucine diluted in DME supplemented with 10% FBS for 30 min. The cells were then washed with ice-cold serum-free DME and lysed on the dish using 1 ml ice-cold 10% TCA plus 1 mM DTT and 1 mM cold leucine. Cellular protein was precipitated, dissolved in formic acid, and the [^{3}H]leucine incorporation was determined by scintillation counting. The protein concentration was determined using the Coomassie blue plus protein reagent (Pierce Chemical Co.). The total counts per minute of [^{3}H]leucine incorporated per milligram of protein for 30 min was calculated for each sample. Samples were prepared in triplicate. Protein degradation assays were done exactly as described elsewhere (Soucek et al., 1998). Cells were treated with 100 μg/ml cycloheximide, and protein abundance followed by Western blotting.

Growth Assays

Five sets of triplicate dishes of cells were plated at 5×10^4 in 35-mm dishes. The triplicate sets of each cell type were counted at 12, 24, 48, and 72 h after plating. Cells were dissociated using pancreatase (GIBCO BRL) for 15 min, resuspended in DME, and placed in Eppendorf tubes. Cells were counted directly on a Beckman Coulter counter after dilution in isotonic saline. The data are plotted as cell counts versus time in order to compare the growth rates for the different clones.

GSH Assay

Total intracellular reduced GSH and oxidized GSH (GSSG) were measured as described previously (Tan et al., 1998b). In brief, cells were plated on 60-mm tissue culture dishes at 2×10^5 cells per dish 12 h before adding 2–5 mM glutamate for 10 h and total GSH was assayed. Pure GSH was used to establish a standard curve.

Flow Cytometric Studies

Cells were plated on 60-mm dishes at 2×10^5 cells per dish 12 h before adding 2–5 mM glutamate for 10 h. Samples were then labeled with the fluorescent dyes DCF and Indo-1 to determine ROS production and Ca^{2+} influx, respectively. Samples were prepared as described previously (Tan et al., 1998b).

Results

eIF2α Is Involved in the Oxidative Glutamate Toxicity Pathway

Although a mechanistic outline of oxidative glutamate toxicity-mediated PCD has been developed (Li et al., 1997a,b; Tan et al., 1998a,b), very little is known about the changes in gene expression that are required for this pathway. To identify genes that may be involved in cell death or the protection from cell death, HT22 cells were infected with a cDNA expression library in a retroviral vector, and cells resistant to high concentrations of glutamate were selected. The retroviral library contained sense, antisense, and partial cDNA sequences. Therefore, glutamate resistance could be due to a sense cDNA which when overexpressed causes glutamate resistance. Alternatively, a transcript from an antisense cDNA could interfere with the expression of a gene normally required for cell death, or the product of a partial cDNA fragment may act in a dominant negative manner to block protein function. A fourth alternative is that during retroviral infection, a cDNA is inserted into the genome in a way that disrupts or upregulates the normal expression of a gene that is involved in glutamate-induced cell death. Finally, ploidy sometimes changes in the cells as they divide, and resistant cells may arise independently of the retroviral infection due to loss of chromosomes or chromosome fragments. This constitutes a background level of naturally resistant clones in the genetic screen. HT22 cells were infected with the retroviral cDNA library and selected in 10 mM glutamate for 48 h, a condition where all of the cells normally die. The cDNA inserts in the pool of glutamate-resistant cells were rescued by remobilizing the vector (Miller et al., 1993), and a second round of infection and selection identified 12 genes that play a putative role in oxidative glutamate toxicity. Identical fragments (213 bp) of the gene encoding

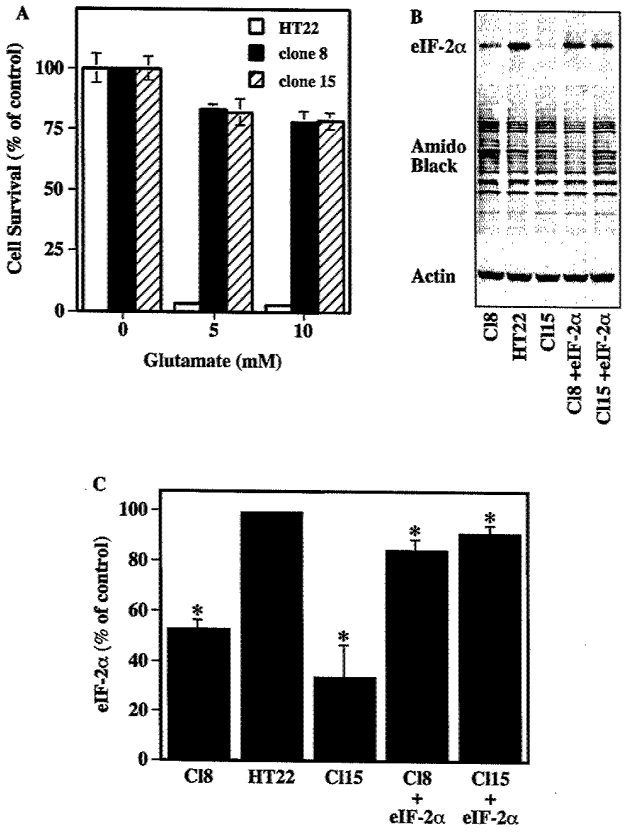

Figure 1. Clones 8 and 15 are resistant to glutamate and express low levels of eIF2α. Glutamate-resistant cells were cloned after infecting HT22 cells with a retroviral cDNA expression library. (A) Cell viability was measured using the MTT assay with wild-type HT22 cells (white bars), clone 8 (black bars), and clone 15 (hatched bars) after a 24-h exposure to 0, 5, and 10 mM glutamate. Samples were measured in triplicate on 96-well plates ($n = 10$). (B and C) Glutamate-resistant clones 8 and 15 have lower levels of eIF2α protein than wild-type HT22 cells which are largely restored by the reintroduction of wild-type eIF2α. (B) eIF2α and actin protein levels were detected by Western blotting of cell lysates (25 μg) from wild-type HT22 cells, glutamate-resistant clones 8 and 15, and clones 8 and 15 transfected with wild-type eIF2α. Loading controls were actin (blot) and amido black staining of the blot. Note that the single band which is reduced in the stained blot in the Cl8 + eIF2α lane is albumin from the serum in the growth medium which is somewhat variable because of washing. (C) The density of each protein band was measured using the program NIH Image, and the average density for each band was plotted relative to eIF2α in wild-type HT22 cells. Identical amounts (±5%) of actin in each lane served as loading controls. The experiment was repeated at least five times with similar results. *Significantly different from HT22 wild-type controls (mean ± SEM, $P < 0.05$).

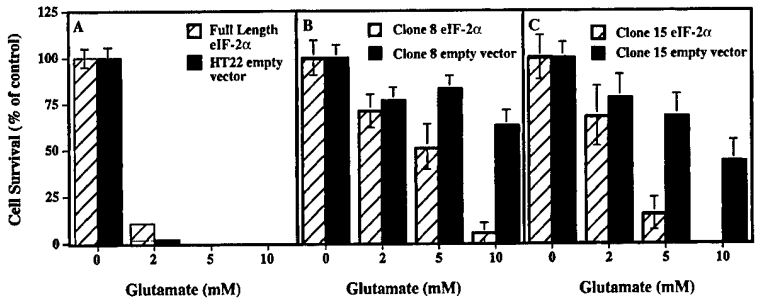

Figure 2. Glutamate-resistant clones 8 and 15 acquire glutamate sensitivity after transfection with full-length eIF2α. Wild-type HT22 cells and glutamate-resistant clones 8 and 15 were stably transfected with an expression construct of eIF2α. (A) The wild-type HT22 cells were unaffected by transfection of eIF2α. (B and C) Resistant clones 8 and 15 when transfected with the eIF2α construct became glutamate sensitive as detected by the MTT assay after 24 h of exposure to 2, 5, and 10 mM glutamate. Samples were measured in triplicate ($n = 3$).

the α subunit of eIF2 (eIF2α) were identified in two separate clones. This gene was chosen for further study because of the requirement for protein synthesis in this form of cell death (Tan et al., 1998a,b). The subclones of HT22, designated clones 8 and 15, are extremely resistant to 10 mM glutamate and were maintained in the presence of 10 mM glutamate (Fig. 1 A). These clones are also resistant to other forms of oxidative stress, including hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide but not to cell death inducers such as TNF-α, anti-FAS antibody, serum starvation, and glucose deprivation (data not shown).

Clones 8 and 15 Cause Glutamate Resistance by Lowering eIF2α Expression

As outlined previously, the introduction of the eIF2α gene fragment into clones 8 and 15 with the retroviral cDNA library could lead to stress resistance by one of several mechanisms. It is unlikely that the eIF2α gene fragment is causing glutamate resistance by disrupting or upregulating a gene whose expression is involved in cell death because the same sequence generates glutamate resistance upon reinfection. This leaves the possibility that the eIF2α cDNA fragment is altering eIF2α expression. Therefore, the two resistant clones and wild-type cells were assayed for eIF2α expression by Western blotting. Although the antibody used for these studies can identify the phosphorylated form of eIF2α (DeGracia et al., 1997), it recognizes both the dephosphorylated and phosphorylated forms of eIF2α in HT22 cells (see Materials and Methods). Using this antibody, it was found that both clones 8 and 15 express lower levels of eIF2α protein (Fig. 1, B and C). Similar results were obtained with another antibody against eIF2α (Ernst et al., 1987).

Since the retroviral expression library contained cDNAs in both the sense and antisense orientations as well as partial fragments of cDNAs, it is likely that an antisense fragment was expressed to downregulate eIF2α expression. The gene fragments that were rescued from clones 8 and 15 are identical and contain a fragment of the eIF2α cDNA from the 3′ end of the full sequence (728–941 bp). Antisense gene fragments from cDNA libraries in retroviral vectors have been used previously to identify physiologically relevant genes (Gudkov and Roninson, 1997). If the downregulation of eIF2α in the resistant clones is responsible for the resistance of the cells to glutamate, then the expression of full-length eIF2α should restore the sensitivity to glutamate. Transfection of full-length eIF2α human cDNA into both clones 8 and 15 restored glutamate sensitivity to both of the clones, whereas the empty vector had no effect (Fig. 2, B and C). The restoration of glutamate sensitivity is not, however, up to the level of wild-type cells at the highest glutamate concentrations, probably because it was only possible to elevate eIF2α to 80–90% of its original level (Fig. 1, B and C). Wild-type HT22 cells remained sensitive to glutamate after being transfected with the full-length eIF2α cDNA (Fig. 2 A). This demonstrates that modulation of eIF2α expression has significant effects on glutamate toxicity in HT22 cells.

eIF2α Phosphorylation Also Mediates Glutamate Resistance

To confirm that the loss of eIF2α activity is linked to glutamate resistance, a second method was employed which utilizes a dominant negative approach to regulate eIF2α function. The phosphorylated form of eIF2α sequesters the guanine nucleotide exchange factor, eIF2β, resulting in a decrease in protein translation (Ernst et al., 1987). The S51D mutant of eIF2α mimics constitutive phosphorylation when serine 51 in eIF2α is replaced with an aspartic acid (Kaufman et al., 1989). The S51A mutant cannot be phosphorylated when serine 51 in eIF2α is replaced with alanine (Pathak et al., 1988). Thus, the S51D mutant inhibits protein synthesis while the S51A mutant prevents the shutdown of protein translation by the phosphorylation of eIF2α. To assay the effect of eIF2α phosphorylation on glutamate sensitivity, wild-type HT22 cells were infected with virus that contained either the S51D or

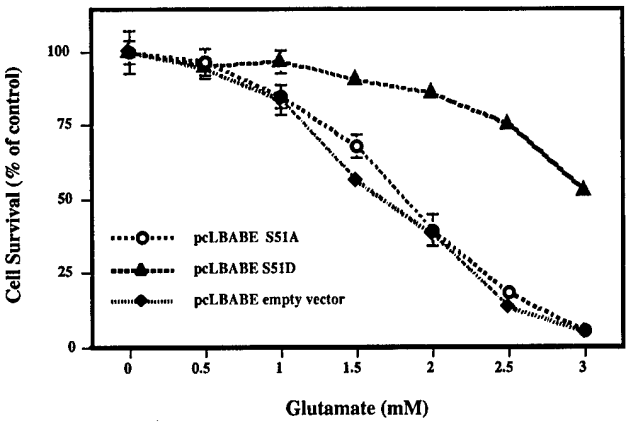

Figure 3. HT22 cells become glutamate resistant when the eIF2α S51D mutant is stably expressed. HT22 cells were infected with virus containing either the pCLBABEpuro empty vector (◆), the S51A mutant of eIF2α (-O), or the eIF2α S51D mutant (▲). Cell viability was measured by the MTT assay. Samples were prepared in triplicate ($n = 4$).

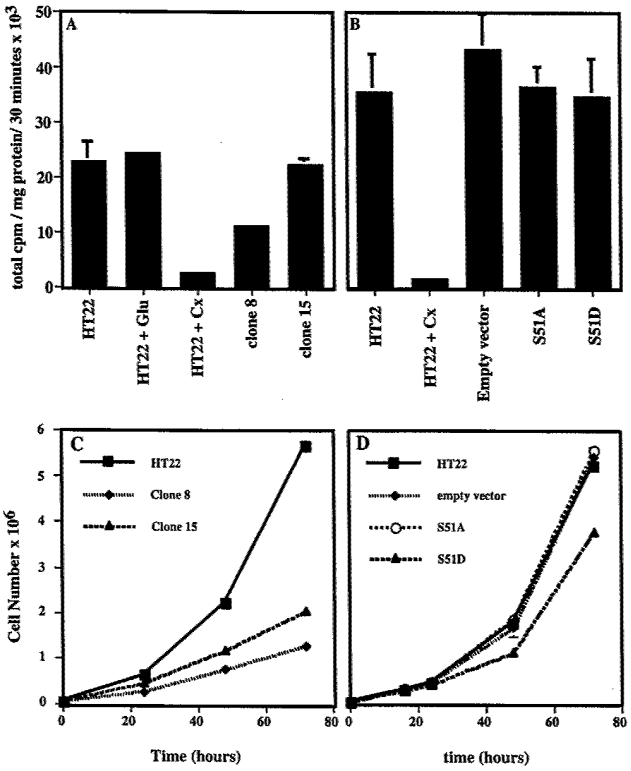

Figure 4. Effect of eIF2α expression on the rate of protein translation. Protein synthesis was measured by [^{3}H]leucine incorporation for 30 min and normalized to total protein. (A) Wild-type HT22 cells (HT22); cells exposed to 5 mM glutamate for 4 h (HT22 + Glu); cycloheximide (HT22 + Cx); and clones 8 and 15. (B) Wild-type HT22 cells and cells infected with either empty vector or the eIF2α phosphorylation mutants S51A or S51D. All assays were performed in triplicate (n = 2). (C) The growth rate is decreased when eIF2α is downregulated or phosphorylated in HT22 cells. Growth rates were measured by counting cells at 0, 24, 48, and 72 h after plating. Wild-type HT22 cells (■); resistant clone 15 (▲); and resistant clone 8 (◆). (D) HT22 cells (■); cells infected with empty vector (◆); eIF2α mutant S51A (○); and mutant S51D (▲). Cell counts at all time points were done in triplicate, and the data are presented as the average ± SEM.

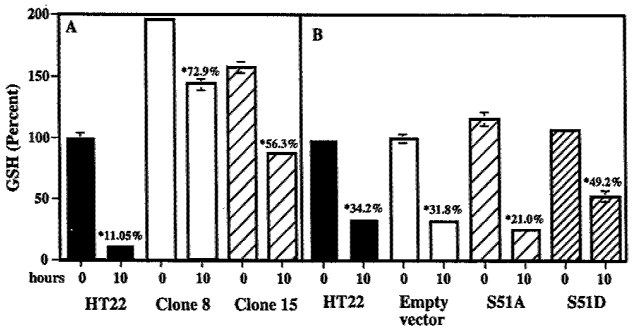

Figure 5. GSH levels in resistant cells. GSH levels were measured in control, untreated cells, and cells exposed to 5 or 2 mM glutamate for 10 h. (A) 5 mM glutamate. Clones 8 (white bars) and 15 (hatched bars) have higher basal GSH levels than wild-type HT22 and only deplete to 72 ± 4 and 56 ± 1% of their basal GSH levels, respectively, with glutamate exposure. (B) 2 mM glutamate. Wild-type HT22 cells (black bars), empty vector infected cells (white bars), and S51A mutant infected cells (hatched bars) show GSH levels that are depleted to 20–30% of basal levels. In the S51D mutant-expressing cells (narrow hatched bars), GSH depleted to only ~50% of the basal level. 100% GSH is defined as the GSH level assayed in the untreated control cells. The numbers above the glutamate exposed bars indicate the percentage of GSH relative to the basal level in the same cell line (n = 3).

S51A mutant or an empty vector, and the cells were tested for glutamate resistance. HT22 cells infected with virus containing the mutant S51D become more resistant to glutamate (Fig. 3). The S51A mutant of eIF2α did not have any effect on the response of the cells to glutamate relative to empty vector (Fig. 3). These data show that the downregulation of eIF2α activity by protein phosphorylation can lead to glutamate resistance and that eIF2α phosphorylation may play an important role in cell death or survival after glutamate exposure. However, we could not directly assay eIF2α phosphorylation after glutamate exposure because none of the available antibodies immunoprecipitate or distinguish phosphorylated from unphosphorylated eIF2α in HT22 cells.

Changes in eIF2α Expression Do Not Affect Translation Rates but Do Slow Growth

To determine if eIF2α downregulation in the glutamate-resistant clones causes a decrease in protein synthesis, protein translation rates were measured in clones 8 and 15 as well as in cells expressing mutants S51A and S51D. By inhibiting translation with cycloheximide, HT22 cells are able to survive in the presence of glutamate for short periods of time (Tan et al., 1998a,b). Therefore, it was important to determine if the inhibition of translation is the sole mechanism by which clones 8 and 15 and the S51D mutant-expressing cell line become resistant to oxidative stress. To measure the rate of translation, cells were labeled with [^{3}H]leucine for 30 min and the total counts per minute of incorporated leucine per milligram of protein calculated. The rate of translation in clone 15 is the same as in wild-type HT22 cells, but it is reduced about twofold in clone 8 (Fig. 4 A). The rate of protein translation is unchanged in HT22 cells after infection with retrovirus containing the eIF2α mutants (S51A and S51D) or empty vector (Fig. 4 B). Similarly, exposure of HT22 cells to glutamate during a 10-h time course does not lead to any significant changes in overall protein translation (data not shown). These data indicate that the inhibition of overall protein synthesis is not the mechanism underlying protection by eIF2α. However, the translation rates do not reflect the growth rates for each clone, as the growth rate of the wild-type HT22 cell line is more than twofold faster than either clone 8 or 15 (Fig. 4 C). HT22 cells infected with the eIF2α mutant S51D also have a slower growth rate than wild-type HT22 cells (Fig. 4 D) even though the protein translation rate of this mutant is the same as that in the wild-type cells (Fig. 4 B). In contrast, the S51A mutant has no significant effect on the translation rate (Fig. 4 B) or the growth rate (Fig. 4 D). These data show that changes in eIF2α expression or activation by phosphorylation may lead to alterations in cell growth but not necessarily translation rates. However, it is possible that although the bulk of protein synthesis is not altered, the synthesis of specific proteins required for cell proliferation and cell death is regulated by altered eIF2α expression or phosphorylation.

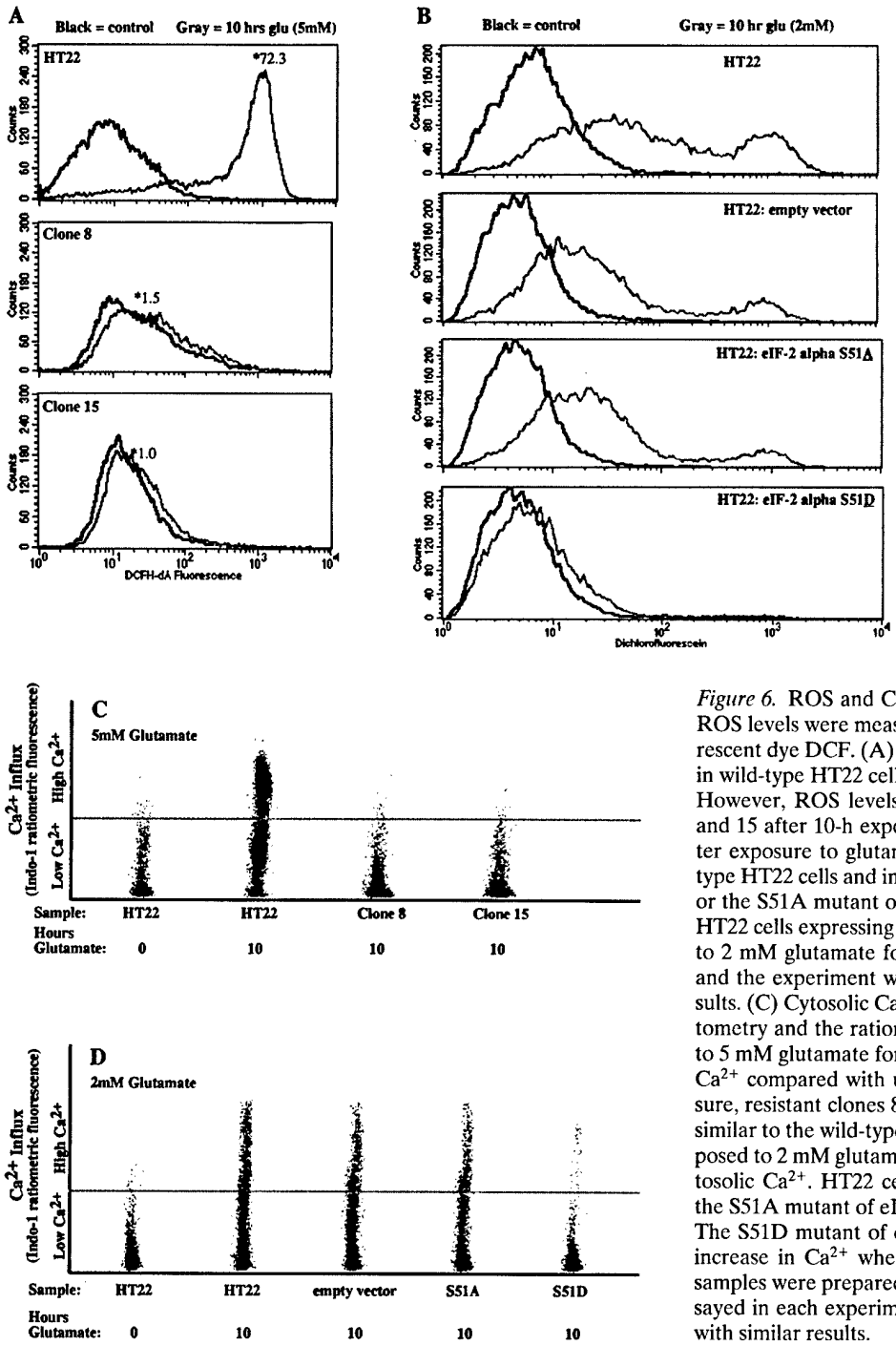

Figure 6. ROS and Ca^{2+} levels after exposure to glutamate. ROS levels were measured by flow cytometry using the fluorescent dye DCF. (A) ROS levels increase 72-fold (gray line) in wild-type HT22 cells exposed to 5 mM glutamate for 10 h. However, ROS levels do not increase in resistant clones 8 and 15 after 10-h exposure to glutamate. (B) ROS levels after exposure to glutamate are increased (gray line) in wild-type HT22 cells and in HT22 cells infected with empty vector or the S51A mutant of eIF2α. ROS levels do not increase in HT22 cells expressing the eIF2α mutant S51D when exposed to 2 mM glutamate for 10 h. 10,000 live cells were assayed, and the experiment was repeated two times with similar results. (C) Cytosolic Ca^{2+} levels were measured using flow cytometry and the ratiometric dye Indo-1. HT22 cells exposed to 5 mM glutamate for 10 h have a large increase in cytosolic Ca^{2+} compared with untreated cells. After glutamate exposure, resistant clones 8 and 15 maintain cytosolic Ca^{2+} levels similar to the wild-type untreated control. (D) HT22 cells exposed to 2 mM glutamate for 10 h have a large increase in cytosolic Ca^{2+}. HT22 cells infected with the empty vector or the S51A mutant of eIF2 also show similar increases in Ca^{2+}. The S51D mutant of eIF2α prevents the glutamate-induced increase in Ca^{2+} when stably expressed in HT22 cells. All samples were prepared in duplicate. 10,000 live cells were assayed in each experiment, and the study was repeated twice with similar results.

eIF2α Expression Alters Glutathione, ROS, and Ca^{2+} Responses to Glutamate

To understand the role of eIF2α in oxidative glutamate toxicity, several parameters of the glutamate response were measured in the resistant clones and the S51A and S51D mutant-expressing cell lines and compared with the wild-type HT22 cells. HT22 cells undergo a rapid depletion of GSH upon exposure to glutamate (Tan et al., 1998b). After 8 h of exposure to glutamate, GSH levels drop below 20% of their normal levels. Comparison of wild-type HT22 cells to glutamate-resistant clones 8 and 15 after 10 h of exposure to 5 mM glutamate revealed that the GSH levels in the resistant cells do not go below 50% of the GSH levels in untreated resistant clones. Furthermore, before glutamate exposure, both cell lines have higher GSH levels than untreated wild-type HT22 cells (Fig. 5 A). The maximal difference in survival between the S51D mutant-expressing cell line and the control HT22 cells is detected at 2 mM glutamate (Fig. 3). When GSH levels in wild-type cells infected with the S51A or S51D mutants or the empty vector are measured after 10 h of exposure to 2 mM glutamate, the S51D mutant cell line shows a decrease to ~50% of the original level compared with the 70% decrease in the wild-type and empty vector–

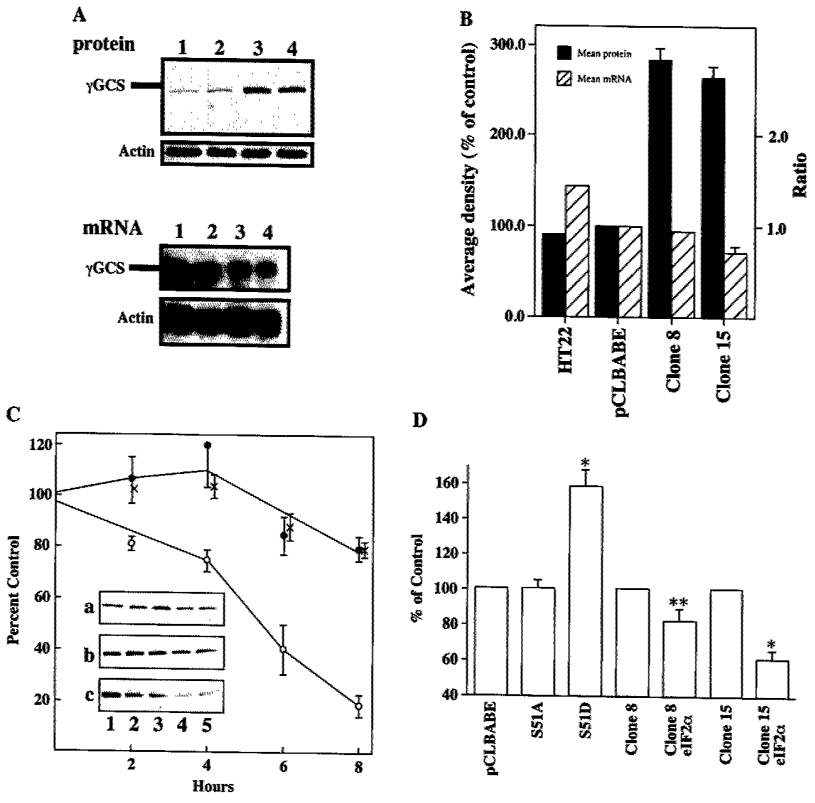

Figure 7. γGCS protein expression is regulated at the level of translation. (A) γGCS, actin protein, and mRNA expression was measured in wild-type HT22 cells (lane 1), cells infected with the empty vector (pCLBABE) (lane 2), and the resistant clones 8 (lane 3) and 15 (lane 4) by Western and Northern blot analysis, respectively. (B) The Western blot from A was analyzed using the program NIH Image to determine the densities of each band. The densities were measured in four experiments, averaged, and normalized first to actin and then to the level of γGCS in pCLBABE, set as 1.0. Actin served as a loading control and showed that there was an equal amount of protein in each lane. Northern blots were quantitated on a PhosphorImager. The ratio of the catalytic subunit of γGCS to actin is presented normalized to γGCS in pCLBABE as 1.0. The results were confirmed by reverse transcription PCR analysis (data not shown). (C) Proteolytic breakdown of γGCS and P27 in wild-type and resistant cells. HT22 cells and resistant clone 15 were treated with 100 μg/ml cycloheximide and the amount of γGCS and P27 was quantitated by Western blot at 2-h intervals. The values are normalized to 0 time and are the mean ± SEM of triplicate experiments. Inset, Western blots of γGCS, wild-type cells (a); γGCS, clone 15 (b); P27, Cl 15 (c). Lanes 1, 2, 3, 4, and 5 are 0, 2, 4, 6, and 8 h after cycloheximide. x, γGCS, wild-type; ●, γGCS Cl 15; ○, P27. (D) Resistant clones 8 and 15 were infected with wild-type eIF2α, the wild-type HT22 clone was infected with S51A or S51D, and the levels of γGCS and actin were determined by Western blotting. The amounts of γGCS and actin were quantitated and the amount of γGCS was normalized to the actin loading control. In each set of cells, the transfected cells were then normalized to γGCS in their parental line pCLBABE, resistant clone 8 or 15, which was set at 100%. The data are presented as the mean ± SEM, $n = 4$. *Significantly different from parental cells ($P < 0.01$); **significantly different from parental cells ($P < 0.05$).

infected cells. On the other hand, the S51A mutant cell line shows a decrease in GSH to ~20% of control levels (Fig. 5 B). This pattern of GSH depletion is consistent with the survival data which demonstrate that although the S51D-expressing HT22 cells are still healthy and dividing after 24 h of glutamate exposure, the other cell lines are dead (Fig. 3). HT22 cells exposed to glutamate for 10 h show a very large increase in ROS which follows the drop in GSH (Tan et al., 1998b). The fluorescent dye DCF was used to determine the levels of ROS production by flow cytometry in the resistant and mutant cell lines after exposure to toxic levels of glutamate. The level of ROS in wild-type HT22 cells after exposure to 5 mM glutamate for 10 h is increased >70-fold (Fig. 6 A). In contrast, the glutamate-resistant cell lines 8 and 15 do not show an increase in ROS above normal levels, and the cells survive and continue to divide (Fig. 6 A). When HT22 cells are exposed to 2 mM glutamate for 10 h, the cells die and there is an increase in ROS, although the DCF intensity is more diffuse than with 5 mM glutamate (Fig. 6 B). The same pattern of increased DCF is seen in cells expressing empty vector and the mutant S51A. However, HT22 cells expressing the mutant S51D have low levels of ROS and were able to survive glutamate treatment (Fig. 6 B). Finally, Ca^{2+} influx was measured in wild-type, resistant, and phosphorylation mutant-expressing cells. Ca^{2+} levels were determined by FACS® analysis using the ratiometric dye Indo-1 (Tan et al., 1998b). After 10 h of exposure to 5 mM glutamate, HT22 cells have much higher levels of Ca^{2+} than untreated controls, whereas resistant cell lines 8 and 15 maintained intracellular Ca^{2+} levels similar to those of the wild-type HT22 cells (Fig. 6 C). The same experiment was performed on the phosphorylation mutant-expressing cell lines exposed to 2 mM glutamate. Ca^{2+} levels increase significantly in the wild-type HT22 cells as well as the empty vector and S51A-expressing HT22 cells. The intracellular Ca^{2+} level in the S51D-expressing cells remained similar to the HT22 cells that were not exposed to glutamate (Fig. 6 D). These data show that both the down-regulation of eIF2α in clones 8 and 15 and the expression of the dominant negative phosphorylation mutant S51D all prevent the decrease in GSH and the increases in ROS and Ca^{2+} normally associated with oxidative stress–induced cell death.

The Inactivation of eIF2α Upregulates γGCS Expression by a Translational Mechanism

Resistant clones 8 and 15 have decreased eIF2α activity and increased basal levels of GSH. Furthermore, the resistant clones and the cells expressing the phosphorylation mutant, S51D, maintain GSH levels 50% of their basal levels after glutamate exposure. To determine if there is a

causal relationship between eIF2α protein levels and GSH production, the expression of the rate-limiting enzyme for GSH synthesis, γGCS, was examined in the wild-type cells and the resistant clones. Protein expression and mRNA levels of the catalytic subunit of γGCS were measured by Western and Northern blotting, respectively. Western blotting shows that the level of the catalytic subunit of γGCS is threefold higher in the resistant clones than in the wild-type HT22 cells (Fig. 7, A and B). In contrast, when both γGCS and actin mRNA were quantitated and their ratio normalized to cells expressing the empty pCLBABE retroviral vector, the amount of γGCS mRNA remained relatively constant (Fig. 7, A and B). To rule out the possibility that eIF2α activity changes the rate of γGCS breakdown, resistant clone 15 and wild-type cells were treated with cycloheximide and the rate of protein loss followed by Western blotting. This method gives values of protein turnover identical to pulse–chase experiments (Soucek et al., 1998). The rapidly turned over cell cycle protein, P27, served as a positive control (Soucek et al., 1998). Fig. 7 C shows that in contrast to P27, γGCS was degraded more slowly but at the same rate in resistant and wild-type cells. These results indicate that a decrease in eIF2α wild-type protein levels leads to an increase in production of the catalytic subunit of γGCS by a translational mechanism, resulting in significantly higher levels of GSH.

If eIF2α directly regulates γGCS expression, then its expression should be upregulated in wild-type cells made resistant by the S51D phosphorylation mutant and downregulated in the resistant cells which were transfected with wild-type eIF2α to render them more sensitive to oxidative stress. Fig. 7 D shows that the levels of γGCS increased ~60% in cells transfected with S51D relative to wild-type cells. In contrast, the expression of γGCS decreased between 20 and 40% in the resistant clones 8 and 15 which already have a high level of γGCS protein when these clones were transfected with normal αIF2α (Fig. 7 B). These data, along with those presented above, strongly suggest that eIF2α expression and activity can directly modulate γGCS protein levels. It is also likely that the expression of additional proteins involved in the resistance to oxidative stress is regulated by eIF2α.

Discussion

The above data show that eIF2α plays a central role in programmed nerve cell death initiated by oxidative stress. Alterations in either the level of eIF2α or its phosphorylation protect cells from glutamate-induced oxidative stress as well as other prooxidant agents. We will first discuss the evidence for the involvement of eIF2α in glutamate-induced cell death, followed by possible mechanisms that eIF2α could use to signal this type of cell death. The potential relevance of eIF2α nerve cell death in PD will also be discussed.

eIF2α Is Specifically Involved in Oxidative Glutamate Toxicity

HT22 glutamate-resistant clones 8 and 15 were derived from a genetic screen after infection with a retrovirus-based cDNA expression library and selection with a high concentration of the prooxidant glutamate. Both clones contain an identical fragment of the gene for eIF2α from the retroviral library. The following evidence shows that eIF2α activity is required for cells to die via oxidative glutamate toxicity and other forms of oxidative stress: (a) eIF2α fragments rescued from the glutamate-resistant cells make wild-type cells resistant to glutamate upon reinfection; (b) Western blotting demonstrates that the eIF2α protein levels in the resistant clones are lower than in wild-type HT22 cells; and (c) eIF2α downregulation alone causes resistance to glutamate since clones 8 and 15, when transfected with full-length human eIF2α, become glutamate sensitive. Since eIF2α regulates the rate of protein translation and cell death requires protein synthesis, it is possible that the inhibition of cell death simply reflects a decrease in the rate of protein synthesis in the resistant cells. However, the decrease of eIF2α in the resistant cells did not necessarily lead to a slower rate of protein synthesis. Although clones 8 and 15 are equally resistant to glutamate, only clone 8 has a rate of protein synthesis which is lower than that in the wild-type cells. In addition, cells infected with the eIF2α phosphorylation mutant S51D, which also induces glutamate resistance, synthesize protein at a rate that is equal to that of the wild-type cells. These results indicate that a decrease in the rate of translation per se does not lead to glutamate resistance. Further evidence that eIF2α phosphorylation plays a key role in determining the fate of the glutamate-exposed HT22 cells is evident when the S51D mutant of eIF2α is expressed in the HT22 cells, resulting in glutamate resistance. The S51D mutant mimics a constitutively phosphorylated form of eIF2α that cannot be dephosphorylated, such that it is able to sequester the guanine nucleotide exchange factor, eIF2B, and inhibit the initiation of protein synthesis (Ernst et al., 1987; Kaufman et al., 1989). Since the infection of HT22 cells with either eIF2α or the phosphorylation mutants leads to overexpression of their respective transcripts but does not alter the overall levels of eIF2α protein (data not shown), the amount of eIF2α protein that is synthesized must be highly regulated. In contrast to our data, the S51D mutant causes apoptosis when transiently transfected into another cell line (Srivastava et al., 1998), presumably because it shuts down protein synthesis. However, in the HT22 cells expressing the S51D mutant, the cells maintain a normal protein synthesis rate, although the growth rate is slower than in the wild-type cells (Fig. 4 D). One explanation for how the HT22 cells infected with the S51D mutant are able to maintain reasonable translation and growth rates is that after infection with the S51D mutant, cells that greatly overexpress the mutant die, whereas the cells that mildly overexpress the mutant protein are able to survive at a slightly slower growth rate. This is likely because the infected cells become less resistant to glutamate with time. Therefore, they probably express sufficient amounts of the S51D mutant to survive glutamate exposure, but the cells that express the lower amounts of the mutant insufficient for survival in glutamate are eventually able to outgrow the other cells when not in the presence of glutamate.

eIF2α Downregulation and the Constitutively Phosphorylated Form of eIF2α Alter the Same Intermediates in the Cell Death Pathway

The observation that the two glutamate-resistant clones selected by expression cloning and the overexpression of the phosphorylation mutant, S51D, produce similar changes in cell physiology during glutamate exposure fur-

ther supports the critical role of eIF2α in the toxicity cascade. These cell lines all exhibit higher GSH levels than controls after glutamate exposure and lower levels of ROS and intracellular Ca^{2+}. GSH levels in wild-type HT22 cells decline to <20% of controls after glutamate exposure, whereas GSH levels in both the resistant clones and the cells expressing the dominant negative S51D mutant drop to <50% of their basal levels. In contrast to control levels, this level of GSH is sufficient to maintain cell viability (Sagara and Schubert, 1998). The basal levels of GSH in the resistant clones were also higher than in the wild-type HT22 cells. It could be argued that the lower rate of translation and cell growth in the resistant cells frees up more cysteine, allowing them to maintain a higher basal level of GSH. However, clone 15 has a very high basal level of GSH but a normal rate of protein synthesis, suggesting that the resistant cells have higher GSH levels because they actively produce greater amounts of this antioxidant.

The above results suggest that the downregulation or phosphorylation of eIF2α during times of stress signals the translation of specific proteins that increase cell survival. Since decreases in either eIF2α activity or protein levels both lead to an increase in GSH, we asked if the rate-limiting enzyme in GSH production, γGCS, was increased in the resistant cells compared with the wild-type HT22 cells. Fig. 7 shows that although the amount of γGCS is increased in the original resistant clones, the γGCS mRNA level remains constant and there is no difference in the rates of γGCS breakdown. In addition, γGCS is upregulated by the phosphorylation mutant, S51D, and downregulated by the introduction of additional eIF2α into the glutamate-resistant clones 8 and 15 (Fig. 7). These data show that eIF2α regulates γGCS expression by a translational mechanism. Amino acid starvation in *Saccharomyces cerevisiae* also causes eIF2α phosphorylation and leads to the selective translation of one specific transcription factor that signals the synthesis of amino acids so that the yeast can survive starvation (Samuel, 1993). A mechanism comparable to that employed by the yeast may be used in HT22 cells when eIF2α activity is low, leading to an increased production of γGCS to promote cell survival. In addition, it was recently shown that another form of stress, the unfolded protein response, causes the phosphorylation of αIF2α and the increased translation of activating transcription factor 4 (Harding et al., 2000).

eIF2α Plays a Unique Role in Programmed Cell Death

There have been several reports that positively link eIF2α to apoptosis: eIF2α phosphorylation by double-stranded RNA–activated protein kinase is the cause of cell death in TNF-α–stimulated cells (Srivastava et al., 1998), and eIF2α is cleaved by caspases after an increase in PKR kinase activity induced by TNF-α or poly(I):poly(C) (Satoh et al., 1999). However, HT22-resistant clones 8 and 15 are not resistant to TNF-α, indicating that they utilize a survival mechanism that is unique to oxidative stress. Ischemia and reperfusion in the rat brain also lead to eIF2α phosphorylation and cell death (DeGracia et al., 1997; Burda et al., 1998). In these cases, it was argued that death signals lead to eIF2α phosphorylation, protein synthesis shutdown, and cell lysis. In contrast, our data show that eIF2α phosphorylation protects cells from death. HT22 cells treated with thapsigargin, a substance shown to cause eIF2α phosphorylation (Prostko et al., 1995), also leads to cell survival after glutamate exposure (data not shown). Finally, although it is generally assumed that any response to central nervous system injury is part of the cell death mechanism, it is equally likely that such a response is a component of a survival pathway (Maher and Schubert, 2000). Therefore, the nature of the stimulus and the extent of eIF2α phosphorylation determine whether eIF2α will be used to prevent or promote cell death. The above experiments link oxidative stress, GSH depletion, and the regulation of γGCS directly to eIF2α and programmed nerve cell death. Markers for both oxidative stress and the depletion of intracellular GSH are found in areas of central nervous system nerve cell death in PD (Sian et al., 1994). However, in both PD and AD large numbers of nerve cells do survive. It is therefore important to understand the mechanisms which lead to resistance to oxidative stress. In the brain, intracellular GSH is the single most important antioxidant, and GSH-peroxidase breaks down H_2O_2 and a variety of organic peroxides, thus protecting cells from oxidative stress. The experiments presented here show that changes in the expression level or phosphorylation of a member of the protein translation complex, eIF2α, can regulate the ability of a nerve cell to deal with oxidative stress. This appears to be primarily done through the regulation of GSH levels, as sustained GSH depletion is the initial event which triggers downstream events such as peroxide accumulation and ultimately cell death. Cells with low amounts of eIF2α or phosphorylated eIF2α maintain high levels of GSH when stressed and do not die. These results point to a central role of eIF2α as a translational switch in the control of oxidative stress within the nervous system. They also suggest a possible therapeutic target for manipulating intracellular GSH levels.

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The Activation of Dopamine D4 Receptors Inhibits Oxidative Stress-Induced Nerve Cell Death

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Oxidative stress is thought to be the cause of nerve cell death in many CNS pathologies, including ischemia, trauma, and neurodegenerative disease. Glutamate kills nerve cells that lack ionotropic glutamate receptors via the inhibition of the cystine–glutamate antiporter x_c^-, resulting in the inhibition of cystine uptake, the loss of glutathione, and the initiation of an oxidative stress cell death pathway. A number of catecholamines were found to block this pathway. Specifically, dopamine and related ligands inhibit glutamate-induced cell death in both clonal nerve cell lines and rat cortical neurons. The protective effects of dopamine, apomorphine, and apocodeine, but not epinephrine and norepinephrine, are antagonized by dopamine D4 antagonists. A dopamine D4 agonist also protects, and this protective effect is inhibited by U101958, a dopamine D4 antagonist. Although the protective effects of some of the catecholamines are correlated with their antioxidant activities, there is no correlation between the protective and antioxidant activities of several other ligands. Normally, glutamate causes an increase in reactive oxygen species (ROS) and intracellular Ca^{2+}. Apomorphine partially inhibits glutamate-induced ROS production and blocks the opening of cGMP-operated Ca^{2+} channels that lead to Ca^{2+} elevation in the late part of the cell death pathway. These data suggest that the protective effects of apomorphine on oxidative stress-induced cell death are, at least in part, mediated by dopamine D4 receptors via the regulation of cGMP-operated Ca^{2+} channels.

Key words: HT22 cells; cell death; apomorphine; apocodeine; dopamine D4 receptors; glutamate; cGMP

Dopamine and its five receptor subtypes play diverse roles in the CNS. Their activation is thought to adversely contribute to several neuropathological disorders, including Parkinson's disease and schizophrenia (Seeman and Van Tol, 1994; Sokoloff and Schwartz, 1995). In addition, dopamine may have a neuroprotective role. Catecholamines, such as dopamine, norepinephrine, and epinephrine, are thought to protect nerve cells at low doses by virtue of their antioxidant activities, but are neurotoxic at high doses, acting as pro-oxidants (Noh et al., 1999). It has also been reported that dopamine receptor agonists have neuroprotective effects that are caused by nonreceptor-mediated mechanisms. For example, bromocriptine and apomorphine act as free radical scavengers (Yoshikawa et al., 1994; Sam and Verbeke, 1995; Grünblatt et al., 1999). In addition, dopamine D3 receptors are not critical for the neuroprotection by the D3 agonist, pramipexole, in 3-acetyl pyridine-treated rats (Sethy et al., 1997). In contrast, some recent reports suggest receptor-mediated mechanisms for protection by dopamine receptor agonists. For example, bromocriptine protects dopaminergic neurons from levodopa-induced toxicity by stimulating dopamine D2 receptors (Takashima et al., 1999).

HT22 cells are immortalized mouse hippocampal cells and can be considered a model of oxidative toxicity on exposure to glutamate. HT22 cells have no ionotropic glutamate receptors (Maher and Davis, 1996), but exogenous glutamate blocks cystine uptake into the cells via the inhibition of the glutamate–cystine antiporter, resulting in decreases in intracellular cysteine and glutathione (GSH). GSH is the major intracellular antioxidant, and its loss leads to an inability of the cell to deal with pro-oxidant conditions (oxidative stress). After GSH depletion, there is an accumulation of reactive oxygen species (ROS) and a large Ca^{2+} influx, resulting in a form of programmed cell death that is distinct from apoptosis (Murphy et al., 1989; Tan et al., 1998a,b; Maher and Schubert, 2000). The accumulation of intracellular Ca^{2+} is by Ca^{2+} influx through cGMP-operated Ca^{2+} channels (Li et al., 1997b). In this study, we examined the neuroprotective mechanisms of dopamine and related compounds on oxidative stress-induced nerve cell death in HT22 cells and primary rat cortical neurons. It is shown that the activation of D4 receptors is responsible for protection from oxidative stress by dopamine and its analogs.

MATERIALS AND METHODS

Materials. The oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). The chemicals used were: [^{3}H]spiperone (specific activity 610.5 GBq/mmol; NEN, Boston, MA); dopamine receptor D4 affinity purified polyclonal antibody (Chemicon, Temecula, CA); 2′,7′-dichlorofluorescein diacetate (DCF), indo-acetoxymethylester (Indo-1), pluronic F-127, and propidium iodode (all from Molecular Probes, Eugene, OR); haloperidol and L745870 (Tocris Cookson, Ballwin, MO); and 8-(4-clorophenylthio) cGMP (pCPT-cGMP), apomorphine, apocodeine, PD168077, U101958, dopamine, and spiperone (Sigma, St. Louis, MO).

Cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The HT22 cells were propagated in DMEM that was supplemented with 10% fetal bovine serum (FBS). Cell survival was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT is taken up by recycling vesicles in which it is reduced and cycled to the extracellular space (Liu et al., 1997). In the HT22 system, it is a valid measure of cell death when compared with trypan blue-based visual counting and colony formation (Maher and

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Davis, 1996). Briefly, HT22 cells were dissociated with pancreatin (Life Technologies, Gaithersburg, MD) and seeded onto 96-well microtiter plates at a density of 2×10^3 cells per well in 100 μl of the same medium. The next day, cells were treated with various reagents according to the experimental design. Twenty hours after the addition of glutamate, the culture medium was replaced with fresh medium because some of the catecholamines directly reduced MTT at the higher concentrations tested. In all cases, parallel dishes containing no cells were used, and for each drug concentration, cells with drug alone (no glutamate) were used to determine whether the drug had a direct interaction with the cell. These controls ensured that no direct reduction of MTT by the catecholamines occurred, and visual counts were done to confirm the MTT data. In some cases, the calcein AM viability assay (Molecular Probes) was used. For the MTT assay, 10 μl of 2.5 mg/ml MTT solution was added then and incubated at 37°C for 4 hr, and 100 μl of solubilization solution (50% dimethylfomamide, 20% SDS, pH 4.8) was added. The next day, the absorption values at 570 nm were measured (Liu et al., 1997). The results are shown as the percentage of the controls specified in each experiment. The primary cortical cells were prepared as described by Sagara and Schubert (1998). In all cases, cell death was confirmed by visual inspection.

Total antioxidant activity assay. Total antioxidant activity was measured using the procedure described by Miller et al., (1993) and expressed as Trolox, a water-soluble vitamin E analog, equivalent antioxidant capacity (TEAC). The TEAC value is the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of sample under investigation. Briefly, 1 ml of reaction mixture including 2.5 μM metmyoglobin, 150 μM 2,2′-azinobis(3-ethylbenzoline 6-sulfonate), 75 μM H_2O_2, and 0.84% sample or Trolox (for standard) in PBS was incubated for 7.5 min at 30°C; then the absorbance at 734 nM was read at 7.5 min. The data are normalized to 1 mM Trolox (TEAC activity).

GSH, ROS, and Ca^{2+} measurements. Total GSH was measured as described by Tan et al. (1998a), using pure reduced glutathione as the standard. ROS and Ca^{2+} measurements were performed as described by Tan et al. (1998a) by flow cytometry. ROS production and intracellular Ca^{2+} were detected using DCF and Indo-1, respectively. Briefly, the cells were incubated with Indo-1 and pluronic F-127 for 25 min at 37°C; then DCF and pancreatin were added, and cells were incubated for 5 min. Cells were collected and washed once in HEPES buffer supplemented with 2% dialyzed fetal bovine serum. Washed cells were resuspended in HEPES buffer and kept on ice until flow cytometric analysis. DCF data were collected with the 475 nm excitation and 525 nm emission wavelengths and plotted as histograms using the data analysis program CELLQuest (Becton Dickinson, Mountain View, CA). Indo-1 data were collected with two emission wavelengths, 410 nm (FL32) and 485 nm (FL4). FL32 and FL4 reflect the fluorescence of Indo-1 with and without bound Ca^{2+}, respectively. The Ca^{2+} concentration is presented as the ratio of FL32/FL4 (Sagara, 1998; Tan et al., 1998a). Data were analyzed from 10,000 live cells as determined by the lack of propidium iodide fluorescence.

Reverse transcription polymerase chain reaction. Total RNA was prepared from HT22 cells and various tissues of mice and then treated with DNase for 30 min at 15°C. Oligonucleotide primers used for the PCR amplification were: D4–1, 5′-CCTTACCCAGCCTCCGGACGA-3′, which corresponds to nucleotides 764–784 of the mouse D4 receptor sequence; and D4–2, 5′-GACACGAAGCAAGCCGGACA-3′, which is complementary to nucleotides 1018–1037 of the same sequence. PCR products were electrophoresed on 2% agarose gels and detected by ethidium bromide.

Western blotting. HT22 cells were collected by scraping in sample buffer. Mouse tissues were homogenized with a Polytron (Kinematica, Basel, Switzerland) for 20 sec in PBS supplemented with a mixture of protease inhibitors (Complete; Roche Molecular Biochemicals, Indianapolis, IN), and then centrifuged at 48,000 × *g* for 30 min at 4°C. The resulting pellets were resuspended in sample buffer (3% SDS, 1% glycerol, 0.5% 2-mercaptoethanol, 0.05% bromophenol blue, and 80 mM Tris-HCl buffer, pH 6.8, with Complete protease inhibitors). The samples were heated for 3 min in boiling water, fractionated on 12% polyacrylamide gels, and electroblotted onto membranes. Dopamine receptor D4 affinity purified polyclonal antibody was used as the primary antibody. Immunoreactive bands were detected with the ECL (Amersham Pharmacia Biotech, Arlington Heights, IL) Western blotting detection reagents.

Binding assays. HT22 cells were homogenized with a Polytron for 20 sec in 15 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, and then centrifuged at 48,000 × *g* for 30 min at 4°C. The resulting pellet, which constitutes the membrane fraction, was resuspended in 50 mM Tris-HCl buffer, pH 7.4. Binding assays were performed as described by Maroto et al. (1995), with minor modifications. Briefly, 0.5 nM [^{3}H]spiperone and the membrane fraction (100–150 μg protein–assay tube) were incubated in 50 mM Tris-HCl buffer, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% ascorbic acid for 2 hr at 4°C. The binding reaction was terminated by rapid filtration through Whatman GF/C filters presoaked in 0.3% polyethylenimine. The filters were immediately washed three times with 4 ml of ice-cold 50 mM Tris-HCl buffer, and radioactivity was measured by liquid scintillation counting. The nonspecific binding was determined in the presence of 100 μM haloperidol. Specific binding was estimated by subtracting nonspecific binding from total binding. Binding assays were done in triplicate. Protein concentration was measured by the Bradford method using bovine serum albumin as the standard.

Statistical analysis. The significance of differences between two groups was assessed by Student's *t* test. The significance of three or more groups was assessed by the Bonferroni test.

RESULTS

Effect of dopamine and related compounds on glutamate-induced cell death

Because there have been suggestions in the literature that catecholamines can protect nerve cells from oxidative stress (Noh et al., 1999; Grünblatt et al., 1999), it was asked whether this phenomenon could be reproduced in a well characterized form of programmed cell death that is initiated by oxidative stress. HT22 cells, which lack ionotropic glutamate receptors, were exposed to increasing concentrations of several catecholamines followed by 2.5 mM glutamate. Cell viability assays performed 20 hr later showed that glutamate alone kills >90% of the cells, whereas dopamine protects HT22 cells in a concentration-dependent manner (Fig. 1*A*). Apomorphine and apocodeine, two other dopamine receptor ligands, are also protective (Fig. 1*A*), whereas epinephrine and norepinephrine are less active. Of the compounds tested, apomorphine was the most effective, followed by apocodeine and dopamine. These compounds did not affect control cell survival, and they also protected cells from higher concentrations of glutamate (data not shown). In addition, apomorphine protects rat cortical neurons lacking ionotropic glutamate receptors from oxidative glutamate toxicity (Fig. 1*B*).

To confirm the data with the MTT assay and to visually present the dramatic effects of the dopamine analogs on rescuing the cells from glutamate toxicity, some of the conditions in Figure 1 were repeated with the cell viability stain calcein AM. Calcein AM is a fluorogenic esterase substrate that passes through the cell membrane and is hydrolyzed inside the viable cell to the green fluorescent product calcein (Vaughan et al., 1995). Then, live cells were quantitated (Fig. 2*A*); photomicrographs of the cells are presented in Figure 2*B*. It is clear that essentially all of the cells were killed by glutamate and that both apocodeine and apomorphine rescue the cells. Quantitation using calcein AM was, within experimental variation, the same as that using the MTT assay (Fig. 1).

Because apomorphine and apocodeine are ligands for dopamine receptors (Van Tol et al., 1991; Seeman and Van Tol, 1994), it is possible that the activation of a dopamine receptor leads to protection. To define the receptor subtype, dopamine receptor antagonists were assayed for their reversal of apomorphine protection in HT22 cells. The protective effects of apomorphine, apocodeine, and dopamine were all inhibited by the D4 antagonists, L745870 and U101958, whereas the weaker protective effects of epinephrine or norepinephrine were not affected by these

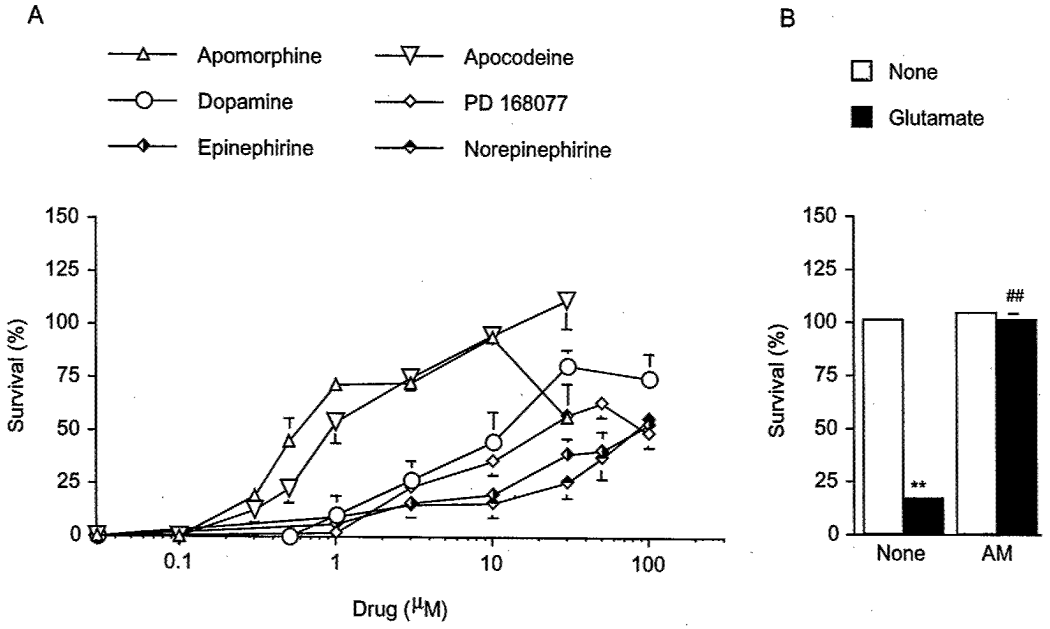

Figure 1. Protective effects of apomorphine and related compounds on glutamate-induced cell death in HT22 cells and primary cortical cells. HT22 (*A*) and 1-d-old primary cortical (*B*) cells were incubated with various drugs and 2.5 mM or 5 mM glutamate, respectively, for 20 hr; then cell survival was measured by the MTT assay. The results are presented as the mean ± SEM relative percentage survival for three or four independent experiments each done in triplicate. *AM*, Apomorphine (1 μM). $^{**}p < 0.01$ (vs no glutamate); $\#\#p < 0.01$ (vs glutamate alone).

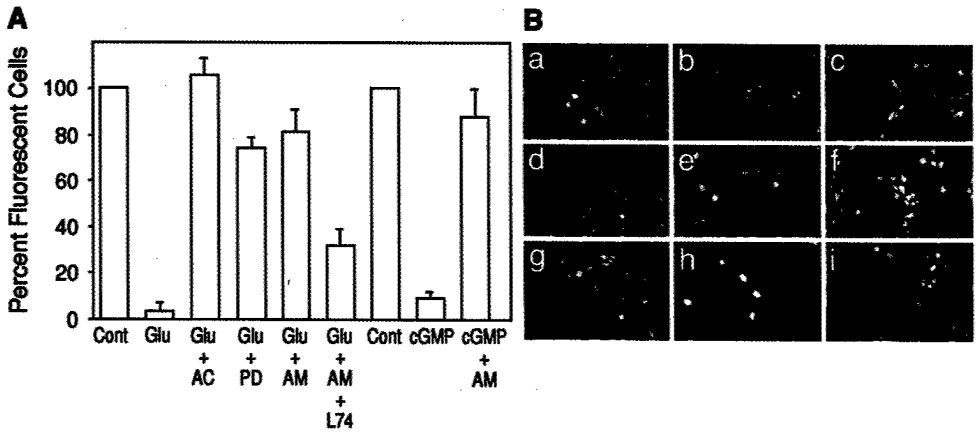

Figure 2. Viability assays using calcein AM. Cells were treated as described below, and viability was determined 20 hr later using calcein AM instead of MTT. *A*, Quantitation of viable cells. Twenty microscopic fields containing between 0 and 80 (control) cells each were scored for viable calcein-positive cells, and the average cells per field in the control (untreated) cultures was given as 100%. The data are presented as the mean cell number relative to control ± SEM. *Cont.*, Control; *Glu*, 2.5 mM glutamate; *AC*, 10 μM apocodeine; *PD*, 50 μM PD168077; *AM*, 1 μM apomorphine; *L74*, 3 μM L745870; *cGMP*, 2.5 mM pCPT-cGMP. The last three columns were from a separate experiment than the first six. *B*, Photomicrographs of calcein-stained HT22 cell cultures after treatment with the reagents described in *A* and Figure 1. The conditions are those described in *A*. *a*, Control; *b*, glutamate; *c*, glutamate plus apocodeine; *d*, glutamate plus PD168077; *e*, glutamate plus apomorphine; *f*, glutamate plus apomorphine plus L745870; *g*, control; *h*, pCPT-cGMP; *i*, pCPT-cGMP plus apomorphine.

antagonists (Fig. 3*A*). The inhibitory effects of dopamine D4 antagonists were not complete, but we could not use higher concentrations because they were toxic. PD168077, a dopamine D4 receptor agonist, also had a significant protective effect on glutamate-induced cell death, and this protective effect was also antagonized by U101958 (Figs. 1, 3*B*). The protective effect of the D4 receptor agonist PD168077 and the antagonistic effect of L745870 are also shown in Figure 2 using the calcein AM viability stain. SCH23390, a dopamine D1 receptor antagonist; sulupiride, a dopamine D2 receptor antagonist; and GR103691, a dopamine D3 antagonist, did not antagonize the protective effects of apomorphine or apocodeine, nor did they affect cell survival in the absence of glutamate (data not shown). These data suggest that the dopamine D4 receptor is involved in the protection of cells from oxidative stress by apomorphine.

Antioxidant activity (TEAC)

It is possible that at least a part of the neuroprotection by apomorphine and related compounds is attributable to their antioxidant activity (Yoshikawa et al., 1994; Grünblatt et al., 1999). To test the antioxidant activities of these compounds, we measured their TEAC values, an index of antioxidant activity *in vitro* (Table 1). Dopamine had the highest antioxidant activity of the compounds shown in Figure 1*A*, and the antioxidant activity of apomorphine tended to be higher than that of epinephrine or norepinephrine. Apocodeine is a weak antioxidant, although it protects cells as well as apomorphine (Fig. 1). In addition, the D4 agonist PD168077, which is also protective, had no antioxidant activity. As shown in Figure 4, the potencies of the protective effects were correlated with antioxidant activities in SKF38393, 7-hydroxy-dipropylaminotetralin (7-OH DPAT), catecholamines,

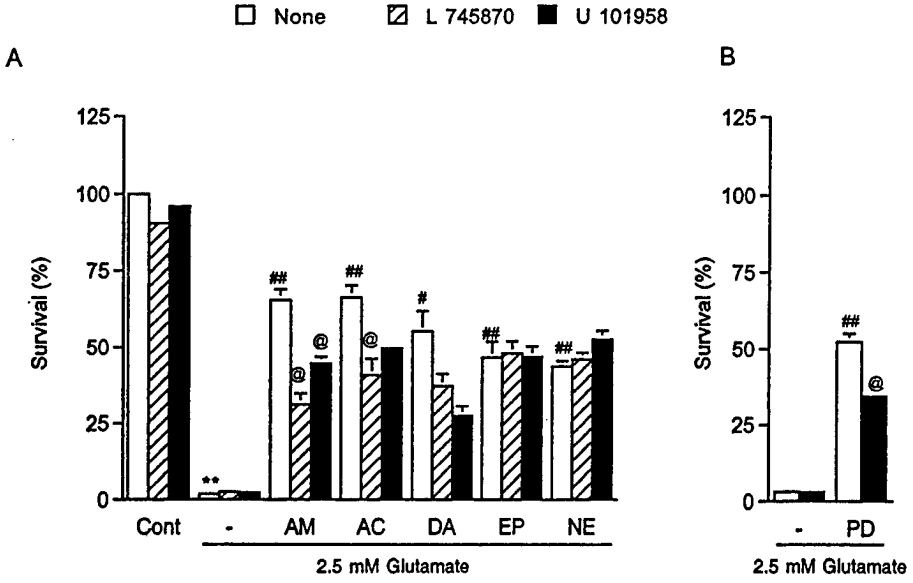

Figure 3. Inhibitory effects of D4 antagonists on the neuroprotective effects by apomorphine and related compounds. *A*, HT22 cells were incubated with glutamate (2.5 mM) and apomorphine (*AM*; 1 μM), apocodeine (*AC*; 1 μM), dopamine (*DA*; 30 μM), epinephrine (*EP*; 100 μM), or norepinephrine (*NE*; 100 μM) in the presence or absence of the D4 antagonists, L745870 (3 μM) or U101958 (10 μM), for 20 hr; then cell survival was measured by the MTT assay. *B*, HT22 cells were incubated with 2.5 mM glutamate and 50 μM PD168077 in the presence or absence of 10 μM U101958 for 20 hr; then cell survival was measured by the MTT assay. The results are presented as the mean ± SEM relative percentage survival for three independent experiments. $^{**}p < 0.01$ (vs control, no glutamate); $\#\#p < 0.01$ (vs glutamate alone); $\#p < 0.05$ (vs glutamate alone); $@p < 0.05$ (vs glutamate in each group). The statistical analysis was performed with the Bonferroni test.

Table 1. TEAC values

Compounds	TEAC (mM)
Apomorphine	1.70 ± 0.07
Apocodeine	0.74 ± 0.05
PD 168077	0.03 ± 0.14
Dopamine	2.40 ± 0.04
SKF 38393	4.11 ± 0.11
7-OH-DPAT	1.34 ± 0.10
Norepinephirine	1.54 ± 0.03
Epinephirine	1.49 ± 0.04
Serotonin	1.86 ± 0.07

TEAC values were measured as described in Materials and Methods. The results are presented as the mean ± SE of four independent experiments.

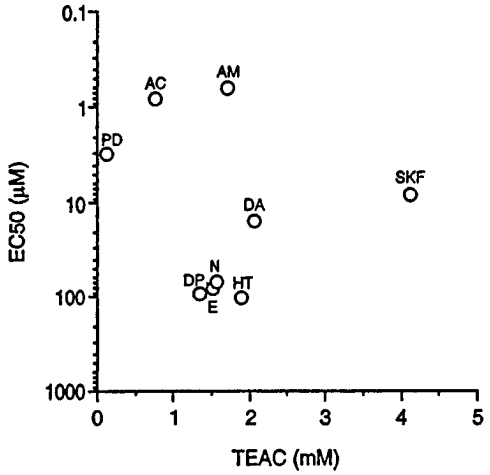

Figure 4. Relationship between the TEAC values and protective effects on glutamate-induced cell death in HT22 cells. TEAC values were obtained from Table 1. EC_{50} values for suppression of 2.5 mM glutamate-induced cell death in HT22 cells were calculated from each concentration–response curve and are presented as the mean of three or four independent experiments. *AM*, Apomorphine; *AC*, apocodeine; *PD*, PD168077; *DA*, dopamine; *SKF*, SKF38393; *DP*, 7-hydroxy-dipropylaminotetralin (7-OH-DPAT); *N*, norepinephrine; *E*, epinephrine; *HT*, serotonin.

and serotonin. SKF38393 is a D1 agonist and has two phenolic hydroxyl groups. 7-OH DPAT is a D3 agonist and has one phenolic hydroxyl group. In contrast, there is no correlation between antioxidant activity and neuroprotection with apomorphine, apocodeine, or PD168077. It is therefore possible that the first group protects cells by virtue of their antioxidant activity, whereas apomorphine, apocodeine, and PD168077 do so by a different mechanism.

Glutathione, ROS and Ca^{2+} levels

Oxidative glutamate toxicity is associated with the depletion of GSH and the elevation of intracellular ROS and Ca^{2+} (Tan et al., 1998a). To determine where in the programmed cell death pathway apomorphine blocked toxicity, we measured intracellular GSH, ROS, and Ca^{2+} levels. Dopamine, apomorphine, and apocodeine did not prevent glutamate-induced glutathione depletion (Fig. 5*A*), showing that this early event in the oxidative stress pathway is not the target for these compounds. In contrast, apomorphine, apocodeine, and dopamine partially inhibited the ROS elevation (Fig. 5*B*). Dopamine showed the strongest inhibition of ROS production, perhaps because of its powerful antioxidant activity. Finally, all of the drugs inhibited Ca^{2+} elevation completely (Fig. 5*C*). Because the influx of Ca^{2+} is a late event in the oxidative glutamate toxicity death program and because some increase in intracellular Ca^{2+} is required for maximum ROS production (Tan et al., 1998a), it follows that apomorphine and apocodeine may inhibit cell death by blocking the influx of Ca^{2+}.

cGMP induced Ca^{2+} influx

In HT22 cells and cortical neurons, cGMP-dependent Ca^{2+} channels are opened near the end of the glutamate-induced cell death pathway (Li et al., 1997b). To test the possibility that apomorphine and apocodeine modulate these Ca^{2+} channels, we examined the effects of these compounds on cell death that was caused by the cell permeable cGMP analog, pCPT-cGMP. pCPT-cGMP caused cell death in a dose-dependent manner (data not shown), and $CoCl_2$, a nonselective Ca^{2+} channel inhibitor, suppressed pCPT-cGMP-induced cell death (Fig. 6*A*). Apomorphine, apocodeine, PD168077, and dopamine also inhibited pCPT-cGMP-induced cell death (Fig. 6*A*), showing that they act at a site downstream from cGMP in the cell death

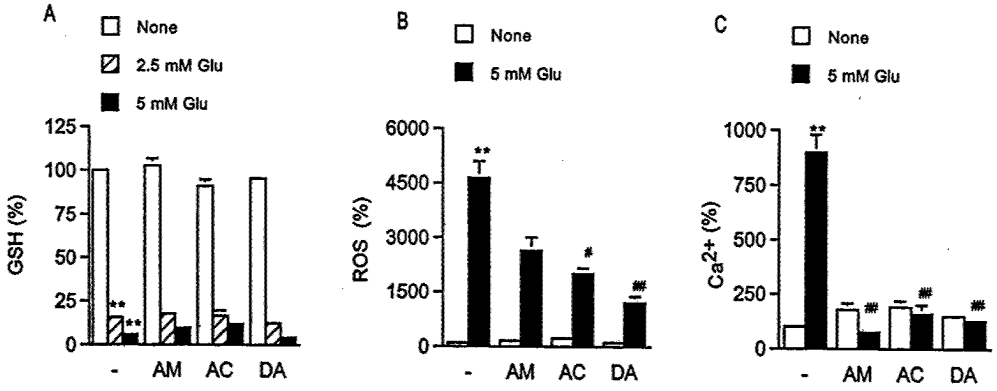

Figure 5. The effects of apomorphine, apocodeine, and dopamine on glutathione (*GSH*) depletion, reactive oxygen species (*ROS*) production, and Ca^{2+} influx by glutamate. Cells were incubated with 2.5 mM (*GSH*) or 5 mM (*GSH*, *ROS*, and *Ca^{2+}*) glutamate and each drug for 8 hr; then GSH, ROS and Ca^{2+} were measured as described in Materials and Methods. GSH was calculated as nanomoles GSH per milligram of protein and presented as a percentage of the control value. ROS and Ca^{2+} were calculated as described in Materials and Methods and presented as a percentage of control. All results are presented as the mean ± SEM for four independent experiments. –, Control; *AM*, apomorphine; *AC*, apocodeine; *DA*, dopamine. $**p < 0.01$ (vs no glutamate); $\#p < 0.05$ and $\#\#p < 0.01$ (vs 5 mM Glu).

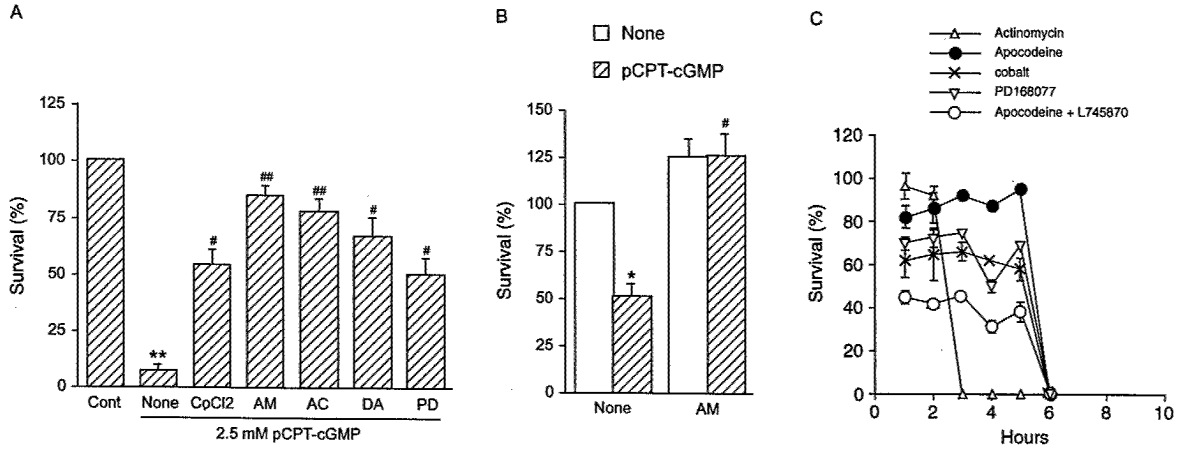

Figure 6. Effects of dopamine receptor ligands on cGMP-induced cell death in HT22 cells and primary cortical neurons. HT22 (*A*) or 1-d-old cultured cortical (*B*) cells were incubated with $CoCl_2$ (50 μM), apomorphine (*AM*; 1 μM), apocodeine (*AC*; 1 μM), dopamine (*DA*; 30 μM), or PD168077 (*PD*; 50 μM), followed by the addition of 2.5 mM pCPT-cGMP. Cell survival was measured by the MTT assay 24 hr later. *C*, Glutamate was added to all samples at 0 time, and 20 μM $CoCl_2$, 1 μM apocodeine, 50 μM PD168077, 1 μM apocodeine plus 3 mM L745870 or 0.1 μg/ml actinomycin D were added at 2 hr intervals up to 10 hr; cell viability was determined after 20 hr. The results are presented as the mean ± SEM relative percentage survival for three independent experiments. $*p < 0.05$ and $**p < 0.01$ (vs no glutamate); $\#p < 0.05$ and $\#\#p < 0.01$ (vs 5 mM glutamate).

pathway. The results with apomorphine using calcein AM are shown in Figure 2. In addition, pCPT-cGMP induced cell death in primary cortical neurons, and these cells were protected by apomorphine (Fig. 6*B*).

If dopamine and its analogs protect cells primarily via the inhibition of Ca^{2+} influx, then it would be predicted that they protect cells from glutamate toxicity when added very late in the cell death program in which the Ca^{2+} influx occurs (Li et al., 1997b; Tan et al., 1998a). In addition, this inhibition should parallel the time course for cobalt protection, and both should occur much later than happens with actinomycin D, for the requirement for mRNA synthesis is a very early event (Tan et al., 1998a). To test this possibility, actinomycin D, $CoCl_2$, the D4 agonist PD168077, apocodeine or apocodeine, plus D4 antagonist L745870 were added to HT22 cells at 2 hr intervals after the addition of glutamate, and cell viability was determined 20 hr later. Figure 6*C* shows that cells are protected from glutamate when $CoCl_2$, apocodeine, or the D4 agonist is added up to 6 hr after glutamate, whereas the protective effect of apocodeine was partially reversed by L745870. Actinomycin D no longer inhibited toxicity when added after 2 hr. These results show that apocodeine protects cells late in the cell death pathway via the activation of D4 receptors and are consistent with both the cGMP and Ca^{2+} influx data.

Dopamine receptors in HT22 cells

The above experiments suggest that HT22 cells have dopamine D4 receptors that mediate the protective response. Three sets of experiments were done to determine whether the HT22 cells express the D4 receptor. D4 receptor mRNA was assayed by reverse transcription polymerase chain reaction (RT-PCR), Western blot experiments were performed to examine protein levels, and ligand binding assays were performed to examine receptor function. In RT-PCR, amplification with D4–1 and D4–2 primers generated a 195 bp cDNA fragment from the HT22 cells, mouse hippocampal RNA, and mouse cortical neuron RNA (Fig. 7*A*). The corresponding band was not detected in mouse liver or kidney. Similarly, Western blots with an antibody specific to D4 receptors showed that HT22 cells, mouse hippocampal membranes, and mouse cortical neurons have dopamine D4 re-

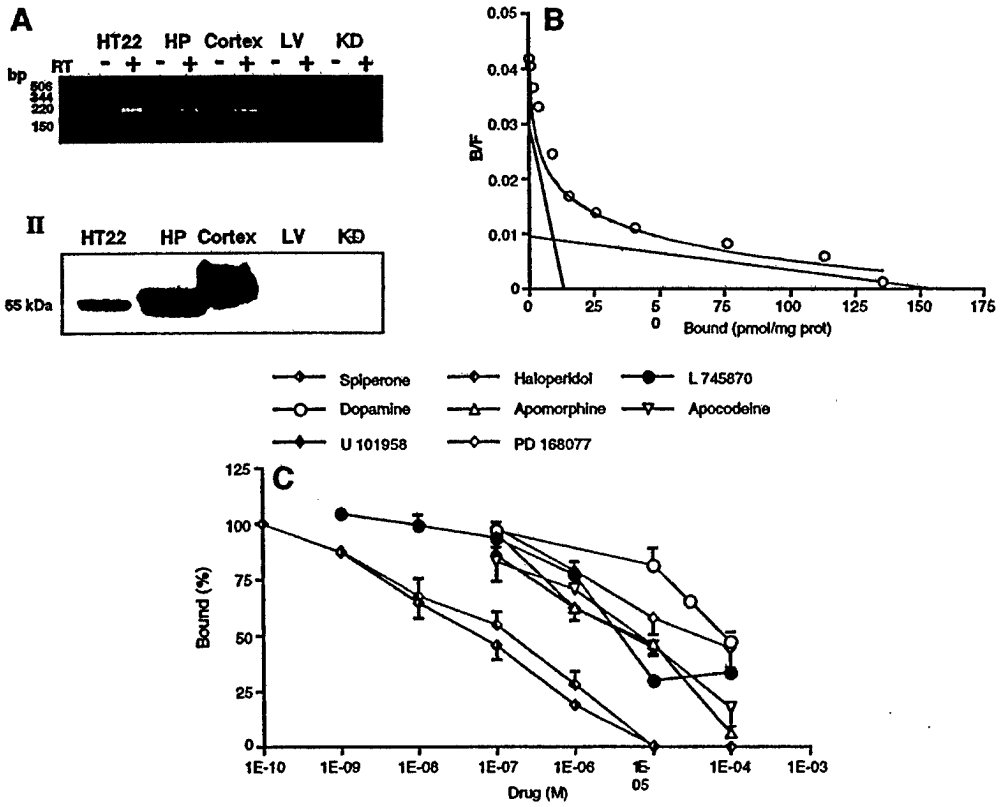

Figure 7. The expression of D4 receptors in HT22 cells. *A*, RT-PCR and Western blot analysis of dopamine D4 receptors in HT22, hippocampal, and cortical cells. DNase-treated total RNA with (+) or without (-) reverse transcription was amplified by PCR. The estimated size of the PCR products was 195 bp. *II*, Lanes of HT22 cell lysates contained 40 μg of protein, and the other lanes contained 20 μg of protein. The estimated molecular weight of the D4 receptor is 55 kDa in HT22 and hippocampal neurons and slightly larger in cortical neurons. *B*, Representative Scatchard plot of [^{3}H]spiperone binding. The binding assay was performed as described in Materials and Methods by using concentrations of spiperone between 0.5 nM and 2.5 μM. *C*, Displacement curves for [^{3}H]spiperone binding. The binding assay was performed as described in Materials and Methods. The results are presented as the mean ± SEM of three to five independent experiments. IC_{50} values are shown in Table 3.

Table 2. K_d and B_{max} values for [^{3}H]spiperone binding

	K_d (nM)	B_{max} (pmol/mg protein)
High-affinity sites	7.21 ± 0.56	28.88 ± 13.08
Low-affinity sites	446.09 ± 177.70	174.02 ± 51.99

Scatchard analysis was performed as shown in Figure 7. The results are presented as the mean ± SE of four independent experiments.

Table 3. IC_{50} values for [^{3}H]spiperone binding

Ligand	IC_{50} (μM)
Spiperone	0.11 ± 0.03
Haloperidol	0.31 ± 0.15
L 745870	3.06 ± 1.21
U 101958	7.66 ± 3.33
PD 168077	26.10 ± 3.12
Apomorphine	5.96 ± 2.15
Apocodeine	6.41 ± 1.49
Dopamine	77.82 ± 10.10

IC_{50} values were calculated from each displacement curve. The results are presented as the mean ± SE of three to five independent experiments.

ceptors (Fig. 7*AII*). In the liver and kidney, this band was not observed. The molecular weight of these proteins was estimated at 55 kDa. These data show that HT22 cells and the tissue from which they were derived, mouse hippocampus, both express D4 receptors.

To further characterize the dopamine D4 receptors in HT22 cells, we performed binding assays. Scatchard plots for the dopamine receptor antagonist [^{3}H]spiperone binding to HT22 cells are curvilinear (Fig. 7*C*), fitting a two site binding model. As shown in Table 2, the K_d values for high- and low-affinity sites are 7.21 ± 0.56 nM and 466 ± 177 nM, respectively, and the B_{max} values are 28.88 ± 13.08 pmol/mg protein and 174.02 ± 51.99 pmol/mg protein, respectively. Displacement experiments showed that various nonselective and D4-selective ligands inhibited [^{3}H]spiperone binding (Fig. 7*C*, Table 3). The most effective displacing agent was spiperone, followed by haloperidol. The IC_{50} value for apomorphine was almost the same as that of apocodeine. The displacing potency of dopamine was the same or weaker than that of the D4 ligands, U101958, L745870, and PD168077.

DISCUSSION

The above data show that the activation of dopamine D4 receptors can play a role in the protection of nerve cells from oxidative stress-induced cell death. This conclusion is based on the following observations. (1) Apomorphine, apocodeine, and dopamine all protect the HT22 mouse hippocampal nerve cell line and rat cortical neurons from oxidative stress induced by oxidative glutamate toxicity (Figs. 1, 2). (2) This protective effect is reversed by D4 antagonists, but not by D1, D2, or D3 antagonists (Fig. 3). (3) A selective D4 agonist, PD168077, also protects neurons from oxidative stress (Figs. 1, 2). (4) The protective effects of the D4 agonists cannot be explained by their inherent antioxidant properties alone (Fig. 4, Table 1). (5) HT22 cells express the mRNA, protein, and physiological binding properties of D4 dopamine receptors (Fig. 7, Tables 2, 3).

It has been suggested that the neuroprotective effects of catecholamines, including dopamine, epinephrine, and norepinephrine are attributable to their antioxidant activities and are not receptor-mediated (Grünblatt et al., 1999; Noh et al., 1999). In the above experiments, it is indeed shown that many catecholamines have antioxidant activity, and that antioxidant activity may be part of the neuroprotective effect of apomorphine. However, D4 receptor mechanisms are more predominant than

antioxidant activities because the protective effect of apomorphine, which is a relatively good antioxidant, is the same as that of apocodeine, which is a relatively poor antioxidant, and stronger than catecholamines, which are also good antioxidants (Table 1). In addition, the D4 agonist PD168077 has no antioxidant activity but is very protective.

It is also known that exogenous dopamine can be neurotoxic. Dopamine is degraded to hydrogen peroxide and dihydroxyphenylacetaldehyde by monoamine oxidase or spontaneously oxidizes to form quinones, semiquinones, and again hydrogen peroxide. Several of these products can lead to the generation of reactive oxygen species such as hydroxyl radicals. Therefore, there has been considerable interest in the potential role of dopamine in CNS ischemia and trauma. Indeed it has been established in several *in vivo* models that dopamine is involved in the cell death pathway. For example, when brain lesions are caused by malonate or 3-nitropropionic acid, two reagents that inhibit energy metabolism, the experimental reduction of dopamine greatly diminishes the extent of damage (Reynolds et al., 1998; Ferger et al., 1999). These results are not, however, at odds with ours because the nature of the insults is quite different (energy deprivation versus oxidative stress) and the levels of multiple toxic agents released from both dying nerve and activated glia are likely to be much higher in severely traumatized CNS tissue than in oxidatively stressed nerve cell cultures. The significance of the dopamine D4 receptor activation may be that it protects cells from gradual changes in low level oxidative stress that occur in mild pathological insults and aging.

The mouse dopamine D4 receptor mRNA is found in the hippocampus and other brain regions, and also in some peripheral tissues such as the adrenal gland and the testes (Van Tol et al., 1991). It is not found in other peripheral tissues, including liver and kidney. Our RT-PCR data from mouse tissues (Fig. 7*A*) are consistent with the published data with respect to the regional specificity of this receptor and size of its mRNA. They also demonstrate that HT22 cells express dopamine D4 receptor mRNA. The mouse D4 receptor has 387 amino acids with a calculated molecular weight of 41,468 (Fishburn et al., 1995; Suzuki et al., 1995). This molecular weight is smaller than the estimated molecular weight from the western blots in this manuscript but is consistent with the apparent molecular weight of the dopamine D4 receptor published by others, probably because of glycosylation (Suzuki et al., 1995; Lanau et al., 1997).

Binding assays also show that dopamine D4 receptors are expressed in the HT22 cells and that apomorphine and apocodeine bind the receptors with higher affinities than dopamine. These data agree with those that show that although apomorphine is a nonselective dopamine receptor agonist, its affinity for D4 receptors is severalfold higher than that of dopamine (Seeman and Van Tol, 1994). In addition, the affinities of apomorphine and apocodeine for the D4 dopamine receptor in HT22 cells are the same as the dopamine D4 antagonist U101958 and higher than the D4 agonist PD168077. These data show that apomorphine, apocodeine, and dopamine can all act as dopamine D4 agonists.

A great deal is understood about the programmed cell death pathway initiated by glutamate in HT22 cells and primary cortical neurons (Maher and Davis, 1996; Murphy et al., 1989; Li et al., 1997a,b; Tan et al., 1998a,b). This knowledge makes it possible to determine at which point in the pathway the drug-induced inhibition of cell death occurs. There are two features of this pathway that are relevant to apomorphine protection. First, maximum ROS production requires the influx of extracellular Ca^{2+} (Li et al., 1997b; Tan et al., 1998a). Second, the influx of Ca^{2+} is initiated by an accumulation of intracellular cGMP via the activation of soluble guanylate cyclase (Li et al., 1997b). The data presented here show that apomorphine, apocodeine, and dopamine inhibit the cell death program at the level of cGMP-gated Ca^{2+} influx. In the presence of these compounds, ROS are elevated but do not reach their maximum levels (Fig. 5). This result is identical to that observed when the synthesis of cGMP is blocked, preventing Ca^{2+} influx, or when the Ca^{2+} channel is blocked by cobalt (Li et al., 1997b; Tan et al., 1998a). The data are therefore consistent with the block being associated with Ca^{2+} influx, a conclusion confirmed by Ca^{2+} imaging (Fig. 5). To more precisely localize the site of protection, it was asked whether apomorphine inhibits cell death that is caused by elevated intracellular cGMP. The elevation of cGMP and the resultant opening of the cGMP-gated Ca^{2+} channels are both necessary and sufficient to cause cell death in oxidative glutamate toxicity (Li et al., 1997b). Figure 6 shows that apomorphine, apocodeine, and dopamine all protect cells from elevated cGMP with a time of action indistinguishable from that of cobalt. Therefore, it is most likely that the activation of D4 receptors inhibits a step downstream of cGMP, probably at the Ca^{2+} channel. Indeed, it has been suggested that the activation of dopamine D4 receptors reduces Ca^{2+} currents (Sokoloff and Schwartz, 1995). The present study shows that apomorphine, apocodeine, and dopamine protect cells from oxidative stress-induced cell death by the inhibition of Ca^{2+} channels through the activation of dopamine D4 receptors.

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Original Contribution

FLAVONOIDS PROTECT NEURONAL CELLS FROM OXIDATIVE STRESS BY THREE DISTINCT MECHANISMS

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Abstract—Flavonoids are a family of antioxidants found in fruits and vegetables as well as in popular beverages such as red wine and tea. Although the physiological benefits of flavonoids have been largely attributed to their antioxidant properties in plasma, flavonoids may also protect cells from various insults. Nerve cell death from oxidative stress has been implicated in a variety of pathologies, including stroke, trauma, and diseases such as Alzheimer's and Parkinson's. To determine the potential protective mechanisms of flavonoids in cell death, the mouse hippocampal cell line HT-22, a model system for oxidative stress, was used. In this system, exogenous glutamate inhibits cystine uptake and depletes intracellular glutathione (GSH), leading to the accumulation of reactive oxygen species (ROS) and an increase in Ca^{2+} influx, which ultimately causes neuronal death. Many, but not all, flavonoids protect HT-22 cells and rat primary neurons from glutamate toxicity as well as from five other oxidative injuries. Three structural requirements of flavonoids for protection from glutamate are the hydroxylated C3, an unsaturated C ring, and hydrophobicity. We also found three distinct mechanisms of protection. These include increasing intracellular GSH, directly lowering levels of ROS, and preventing the influx of Ca^{2+} despite high levels of ROS. These data show that the mechanism of protection from oxidative insults by flavonoids is highly specific for each compound. © 2001 Elsevier Science Inc.

Keywords—Flavonoids, Oxidative stress, Glutamate toxicity, Vitamin E, Reactive oxygen species, Mitochondria, Glutathione, Neuronal cells, Quercetin, Buthionine sulfoximine, Apoptosis, Free radicals

INTRODUCTION

Flavonoids (Fig. 1) are a family of diphenylpropanes found ubiquitously in fruits and vegetables as well as in food products and beverages derived from plants such as olive oil, tea, and red wine. The flavonoid content in fruits and vegetables can be as high as 300 mg/kg fresh weight [1–3] and humans consume between 20 and 80 mg flavonoids per day, an intake higher than that for vitamin E [4]. Flavonoids are also considered to be the active ingredients in some medicinal plants [5,6]. Because ingested flavonoids enter the plasma [7–10] to elevate the redox and antioxidant levels [11,12], the effects of flavonoids may be physiologically significant. Indeed, flavonoids and other plant-derived polyphenolic compounds have recently captured public interest because flavonoids in tea, fruits, and vegetables reduce the risk of cardiovascular diseases [13,14]. Similarly, what is known as the "French Paradox" points to possible benefits of the Mediterranean diet, which includes high amounts of fresh fruits and vegetables as well as red wine [15].

These physiological benefits of flavonoids are generally thought to be due to their antioxidant and free radical scavenging properties, even though flavonoids display other biological activities [16–18]. The efficacy of flavonoids has been studied most extensively in cell-free systems in which reactive oxygen species (ROS) are produced either chemically [19,20] or by radiolysis [21], and the elimination of ROS by flavonoids is monitored directly or by measuring peroxidation levels of lipids [22–24] or LDL [7,25]. These experiments test the ability of flavonoids to act as antioxidants in an aqueous environment and have been very successful in elucidating relationships between activity and structure of flavonoids in solution [7,26]. It is difficult, however, to extend these results to cells that are under oxidative

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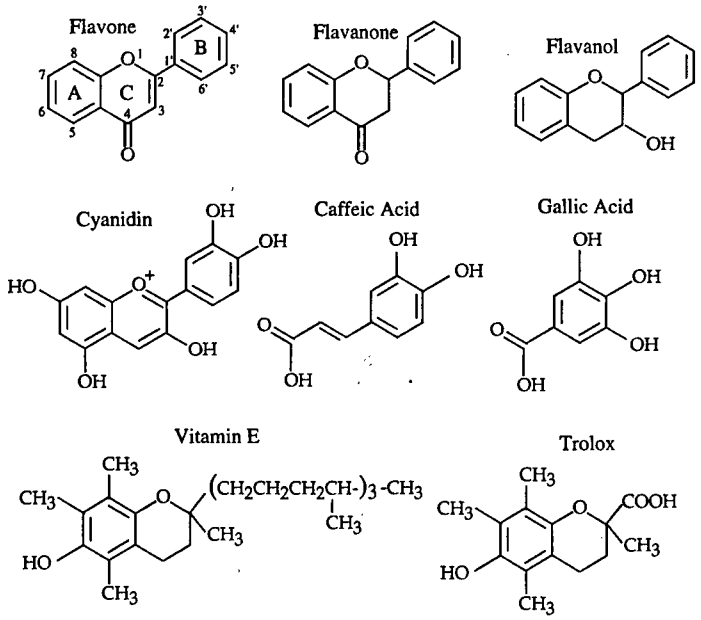

Fig. 1. Chemical structures of flavonoids and phenolic compounds used in this study. The position of hydroxyl groups and common names are listed in Table 1.

stress because the cellular structure encompasses both hydrophilic and hydrophobic compartments such as the cytoplasm and the various membrane systems, respectively.

While the sources of ROS vary in biological systems, significant levels of ROS are produced within cells [27]. For example, mitochondria produce ROS during normal respiration and mitochondrial derived ROS are required for many forms of cell death [28–31]. In neurons and other cell types, extracellular glutamate triggers oxidative stress and subsequent programmed cell death as the consequence of ROS generated from mitochondria [32–37]. Cellular markers and the precise time course of this cascade have been studied extensively in the mouse hippocampal cell line HT-22 [30,38–42]. These cells lack ionotropic glutamate receptors, and glutamate in this system inhibits cystine transport and leads to GSH depletion. Subsequently, ROS produced by mitochondria accumulate to levels 50- to 100-fold higher than the control, causing an increase in Ca^{2+} influx, and finally cell death [30,43]. The importance of oxidative stress in this cascade is supported by the observation that the exogenous addition of an antioxidant such as vitamin E protects cells from glutamate [30,33,38]. Therefore, this system was used to test the protective efficacy of plant-derived flavonoids against cellular oxidative stress.

We show that many flavonoids and related polyphenolic compounds protect HT-22 cells and rat primary neurons from oxidative stress caused by glutamate. The relationship between structure and protective efficacy of flavonoids was also determined. Additionally, flavonoids protect neuronal cells from oxidative injury caused by homocysteic acid (HCA), cystine deprivation, buthionine sulfoximine (BSO), hypoglycemia, and hydrogen peroxide (H_2O_2). Finally, three distinct mechanisms of protection by flavonoids can be identified: the alteration of GSH metabolism, quenching of ROS, and the inhibition of Ca^{2+} influx that signals the last step in the cell death cascade induced by glutamate.

MATERIALS AND METHODS

Flavonoids were purchased from Alexis (San Diego, CA, USA), Aldrich (Milwaukee, WI, USA), or CalBiochem (San Diego, CA, USA). 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-dA), dihydrorhodamine, Fluo-3, Pluronic 127, indo-1, and Fura Red were purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

Cell culture

Fetal bovine serum (FBS) and dialyzed FBS were from Irvine Scientific (Irvine, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was made according to the original procedure [44]. HT-22 cells [38,39] were derived from the mouse hippocampus [45], and were grown on tissue culture dishes (Falcon, Indianapolis, IN, USA) in DMEM supplemented with 10% FBS. Pancreatin (Life Technologies, Rockville, MD, USA)

was used to dissociate cells from culture dishes. Short-term primary cortical neurons from 17 d old rat embryos were prepared according to Abe and Kimura [46]. The primary cells were used for experiments within 3 d after plating. Except where noted, all experiments were done with HT-22 cells.

Cytotoxicity assay

Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to a standard procedure [47–49]. Results obtained from the MTT assay directly correlated with the extent of cell death as confirmed visually by trypan blue staining. The culture medium for both primary neurons and HT-22 both contained 260 μM cystine and 1 mM Mg^{+2}. H_2O_2 toxicity was performed according to conditions established previously [30,42].

Total intracellular GSH/GSSG

Cells were washed twice with ice-cold PBS, collected by scraping, and lysed with 3% sulfosalicylic acid. Lysates were incubated on ice for 10 min and supernatants were collected after centrifugation in an Eppendorf microfuge. Upon neutralization of the supernatants with triethanolamine, the concentration of total glutathione (reduced and oxidized) was determined by the method described originally by Tietze [50] and modified by Griffith [51]. Pure GSH was used to obtain the standard curve. The protein content of each sample was determined using a kit from Pierce (Rockford, IL, USA) with BSA as a standard.

Reactive oxygen species (ROS) levels

Intracellular accumulation of ROS was determined with H_2DCF-dA [52]. This nonfluorescent compound accumulates within cells upon de-acetylation. H_2DCF then reacts with ROS to form fluorescent dichlorofluorescein (DCF) [53]. HT-22 cells were dissociated from tissue culture dishes with pancreatin in DMEM in the presence of 10 μM H_2DCF-dA for 10 min at 37°C, washed once with room temperature DMEM (without phenol red) supplemented with 2% dialyzed FBS, and resuspended in 750 μl of the same solution containing 2 μg/ml propidium idodide (PI). The use of pancreatin did not affect the outcome of flow cytometric experiments as confirmed by fluorescence microscopy. Flow cytometric analysis was performed using a FACScan instrument (Becton-Dickinson, San Jose, CA, USA) with the excitation wavelength (λ_{ex}) of 475 nm and the emission wavelength (λ_{em}) of 525 nm. Data were collected in list mode on 10,000 cells after gating only for characteristic forward versus orthogonal light scatter and low PI fluorescence to exclude dead cells. Median fluorescence intensities of control and test samples were determined with CellQuest software (Becton-Dickinson).

Intracellular calcium

The intracellular level of Ca^{2+} was determined using Fluo-3 acetoxymethylester (AM) or Fura Red as described elsewhere [30,42,54]. The membrane permeable Fluo-3 AM is converted to Fluo-3 upon de-acetylation within the cell and Fluo-3 increases its green fluorescence upon Ca^{2+} binding. Cells were loaded with 0.5 μM Fluo-3 AM in the presence of 0.005% Pluronic 127 for 20 min at 37°C, washed once with room temperature DMEM (without phenol red) supplemented with 2% dialyzed FBS, and resuspended in 4 ml of the same solution containing 2 μg/ml PI. A Nikon light/fluorescence microscope equipped with 32 mm water-immersible objective lens was used to determine Fluo-3 fluorescence and photographed with Kodak Ektachrome 400HC.

Determination of the trolox equivalent activity concentration (TEAC)

Values of TEAC for flavonoids and other phenolic compounds were determined according to Rice-Evans and Miller [55]. Briefly, the inhibition of the absorbance of the radical cation formation of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) was monitored at 7.5 min in a Dulbecco's phosphate buffered saline (PBS) containing 150 μM ABTS, 2.5 μM metmyoglobin, and 75 μM H_2O_2 at 734 nm. The TEAC value of an antioxidant was calculated experimentally using a 0.3 mM Trolox solution and normalizing the data to 1.0 mM Trolox (i.e., 0.3 mM Trolox was used in the experiments and the data multiplied by 3.3 to convert to 1 mM equivalents).

Statistical analysis

Experiments presented were repeated at least twice with triplicate samples. The data are presented as means ± SEM.

RESULTS

Protection from glutamate toxicity by flavonoids

The mouse hippocampal cell line HT-22 has been used to elucidate sequential cellular events during the programmed cell death cascade triggered by glutamate toxicity [30,38,42]. HT-22 cells lack ionotropic glutamate receptors that could mediate excitotoxicity, and are

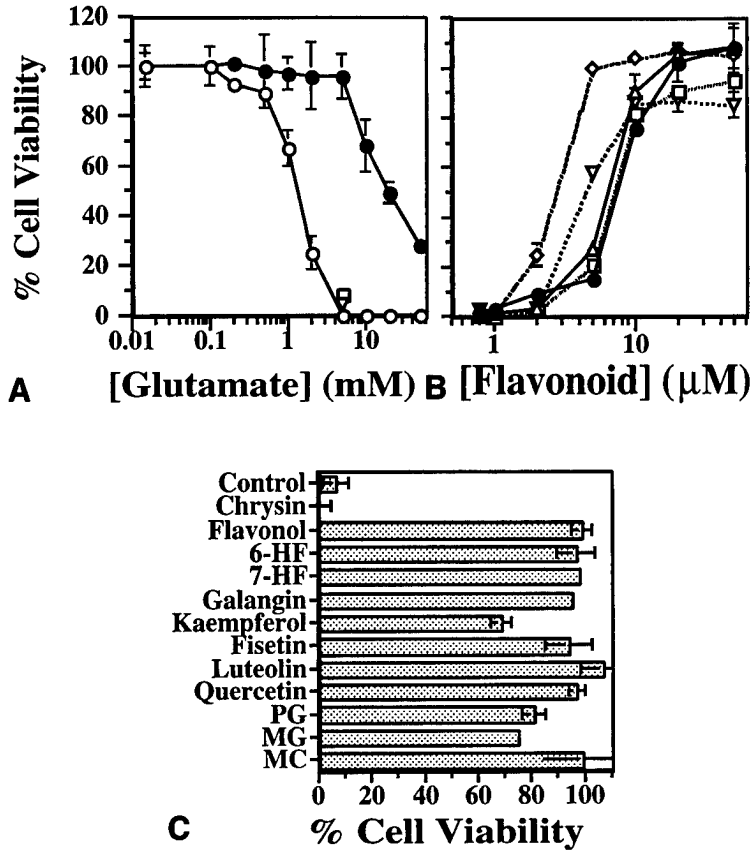

Fig. 2. Cytotoxic response of HT-22 cells to glutamate and protection by flavonoids. Exponentially dividing HT-22 cells were dissociated with pancreatin and plated into a 96 well microtiter plate in 100 μl DMEM with 10% dialyzed FBS. (A) 12 h later cells were exposed to the indicated concentrations of glutamate in the presence of no flavonoids (○), 10 μM galangin (●), 10 μM chrysin (1 point, 5 mM) (▽) or 10 μM catechin (1 point, 5 mM) (□) for 24 h and cell viability was assessed with the MTT assay as described in Materials and Methods. (B) Cell viability was assessed as above in the presence of 5 mM glutamate and the indicated concentrations of galangin (●), flavonol (Δ), 6-HF (□), luteolin (△), or quercetin (◇). (C) Rat primary cortical neurons were exposed to 5 mM glutamate for 24 h in the presence of 10 μM flavonoids as indicated. Cell viability was determined with the MTT assay.

completely killed by 5 mM glutamate in 24 h with a half maximal concentration 1.5 mM (Fig. 2A). This toxicity is via the inhibition of cystine uptake by glutamate, resulting in the depletion of glutathione (GSH), and a form of programmed cell death called oxidative glutamate toxicity [33]. To determine if flavonoids are effective protectants against this form of oxidative injury, HT-22 cells were exposed to various concentrations of glutamate for 24 h in the presence of 10 μM of the flavonoids galangin, chrysin, or catechin, and cell viability was determined by the MTT assay. Galangin protects HT-22 cells from glutamate, but chrysin and catechin are ineffective (Fig. 2A). To determine the effective concentrations of flavonoids, HT-22 cells were exposed to 5 mM glutamate in the presence of various concentrations of a flavonoid and cell viability was determined as above. Galangin, flavonol (3-hydroxyflavone), and 6-hydroxyflavonol (6-HF) all have the effective half maximal concentrations (EC_{50}) of about 7 μM, and the maximum protection is observed with concentrations higher than 10 μM (Fig. 2B). Quercetin and luteolin have the EC_{50} of 2.2 μM and 4 μM, respectively (Fig. 2B). The protection by these flavonoids is not transient: the neuronal cells are viable in toxic doses of glutamate for at least 4 d if protective flavonoids are continuously present (data not shown).

Unexpectedly, the number of hydroxyl groups in a flavonoid did not correlate with the protective efficacy [26]. For example, flavonol with one hydroxyl group is as effective as galangin (three hydroxyl groups) while catechin (five hydroxyl groups) is totally ineffective (Fig. 2A and B). Therefore, the efficacy of several commercially available flavonoids and phenolics was determined to elucidate structural requirements for the protection of HT-22 cells from glutamate toxicity (Table 1). Some flavonoids (α- and β-naphthoflavone) and flavonoid analogs (chalcones and coumarins) are not protective (data not shown), while other phenolic compounds are quite active (Fig. 1; Table 1). The results and the structural requirements for protection will be summarized in the Discussion. The flavonoids that protect HT-22 cells from

Table 1. The Protective Efficacy of Various Flavonoids

Flavonoid[a]	Free hydroxyl positions	Common name	EC_{50} (μM)	TEAC[b] (mM)
Flavone and flavonol	—		No[c]	0.30 ± 0.10
	3	flavonol	6	1.06 ± 0.18
	3-methoxy		No	−0.01 ± 0.07
	6		No	0.95 ± 0.18
	6-methoxy		No	−0.04 ± 0.05
	7		No	−0.04 ± 0.02
	3,6		6	2.06 ± 0.20
	3,7		6	1.65 ± 0.12
	5,7	chrysin	No	2.52 ± 0.12
	3,5,7	galangin	6	2.08 ± 0.11
	5,6,7	baicalein	1	1.22 ± 0.17
	4′,5,7	apigenin	No	2.80 ± 0.50
	3,4′,5,7	kaempferol	10	1.45 ± 0.08
	3′,4′,5,7	luteolin	5	2.48 ± 0.23
	3,3′,4′,7	fisetin	3	2.80 ± 0.06
	3,3′,4′,5,7	quercetin	3	4.84 ± 0.45
	3,3′,4′,5,7[d]	rutin	No	2.67 ± 0.24
	2′,3,4′,5,7	morin	70	2.60 ± 0.24
	3,3′,4′,5,5′7	myricetin	No	3.08 ± 0.46
Isoflavone	4′,5,7	genistein	No	2.96 ± 0.49
Flavonone	—		No	0.01 ± 0.12
	6		No	1.47 ± 0.14
	4′,5,7	naringenin	No	2.48 ± 0.24
	3,3′,4′,5,7	taxifolin	No	3.09 ± 0.58
Flavanol	3,3′,4′,5,7	catechin	No	3.42 ± 0.43
	3,3′,4′,5,7	epicatechin	No	3.16 ± 0.58
Anthocyanidin	3,3′,4,29,5,7	cyanidin	No	3.63 ± 0.65
Vitamin E			0.2	1.1 ± 0.14
Vitamin E acetate			1.5	ND
Vitamin E succinate			12	ND
Trolox			50	1
Probucol			10	0.01 ± 0.15
Caffeic acid	3,4		No	1.20 ± 0.05
Methyl caffeate (MC)	3,4		0.5	1.21 ± 0.15
Gallic acid	3,4,5		No	2.69 ± 0.41
Methyl gallate (MG)	3,4,5		8	2.14 ± 0.30
Propyl gallate (PG)	3,4,5		0.2	2.42 ± 0.08
Catechin gallate (CG)			No	5.12 ± 0.55
Resveratrol	3,4′,5		8	2.88 ± 0.15
PBN[e]			No	ND

Half maximal effective concentrations (EC_{50}) were determined by exposing HT-22 cells to 5 mM glutamate in the presence of a flavonoid and cell viability was assessed as in Fig. 2. Additionally, TEAC values were determined as in Materials and Methods in two triplicate experiments and means ± SEM were reported.

[a] See Fig. 1 for chemical structures.

[b] TEAC is defined as the concentration of Trolox solution equivalent to a standard concentration of the compound in question and the mean ± SEM was presented.

[c] No—These compounds did not protect cells significantly from 5 mM glutamate at 50 μM or lower concentrations.

[d] Rutin is quercetin with the substitution on the C3 hydroxyl group. ND = not determined.

[e] N-*tert*-butyl-α-phenylnitrone.

glutamate toxicity are also effective protectants of primary rat cortical neurons from glutamate toxicity (Fig. 2C), showing that the response of HT-22 neurons to oxidative stress is typical of cortical neurons.

Protection from other forms of oxidative stress by flavonoids

It was asked next if flavonoids protect HT-22 cells from other forms of oxidative injury. Cystine deprivation caused by excess glutamate is also induced by homocysteic acid (HCA) or by excluding cystine from the culture medium [33,39,42]. In contrast, BSO lowers levels of intracellular GSH by inhibiting the GSH synthetic enzyme γ-glutamylcysteine synthetase (γ-GCS) [56]. Fifty μM BSO lowers GSH levels to near zero in HT-22 cells [30,40,41], as does cystine deprivation and 2 mM HCA, resulting in neuronal death [42,43]. In all of these cases galangin at 10 μM protects HT-22 cells (Fig. 3A). All of the other flavonoids that protect HT-22 cells from glutamate are also effective against HCA, cystine deprivation, and BSO (data not shown).

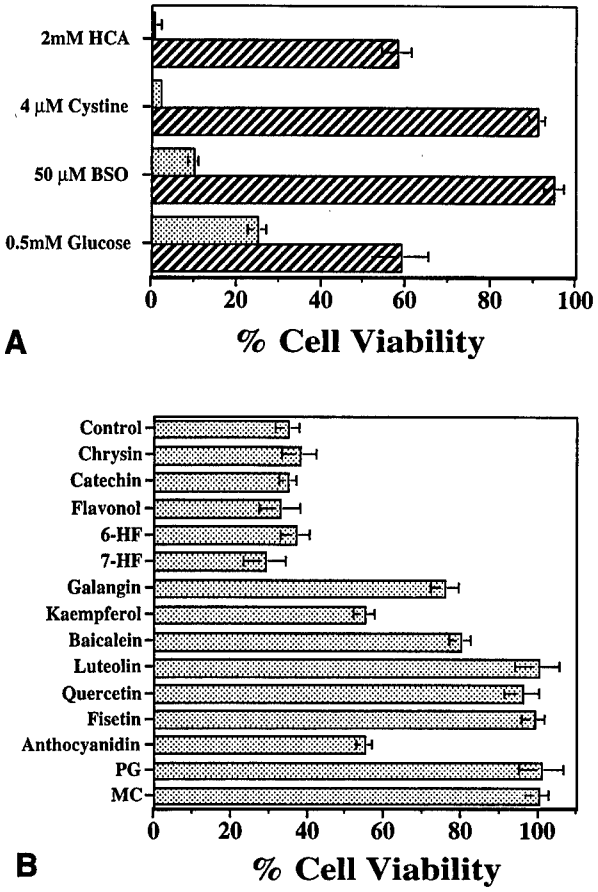

Fig. 3. Protective effects of flavonoids to oxidative stress. HT-22 cells were plated into a 96 well microtiter plate as in Fig. 2. 12 h later the cells were exposed to the indicated conditions for 24 h in the presence (hatched bars) or absence (dotted bars) of 10 μM galangin (A). Cells were exposed to 1 mM H_2O_2 for 8 h in the presence of a flavonoid (10 μM) as indicated (B). The cells were then cultured for an additional 16 h in fresh media and cell viability was determined by the MTT assay.

Oxidative stress is also induced during glucose starvation, or hypoglycemia [57,58]. HT-22 cells die when the glucose concentration of the culture media is decreased from the normal 50 mM to 0.5 mM [49]. Galangin increases cell viability from 20 to 60% in hypoglycemic injury (Fig. 3A). Other flavonoids found to be protective in glutamate toxicity (flavonol, 6-HF, 7-HF, galangin, baicalein, kaempferol, luteolin, fisetin, and quercetin) also effectively protect HT-22 cells from hypoglycemia and the oxidative injuries listed above (data not shown).

The direct addition of the peroxidizing agent H_2O_2 also induces neuronal death. Therefore, the protective efficacy of flavonoids was assessed by exposing HT-22 cells to H_2O_2 for 24 h. Luteolin, quercetin, fisetin, propyl gallate (PG), and methyl caffeate (MC) almost completely block H_2O_2 toxicity (Fig. 3B). Galangin and baicalein protect HT-22 cells to 70 to 80% viability,

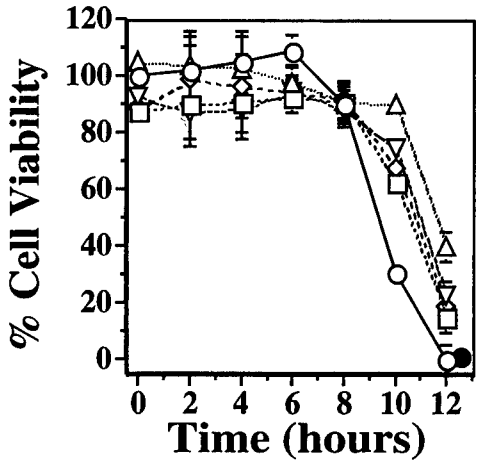

Fig. 4. Time course of glutamate toxicity. HT-22 cells were plated into 96 well microtiter plates as in Fig. 2 and 12 h later exposed to 5 mM glutamate. 24 h glutamate exposure resulted in less than 3% survival (●). For one sample (○), glutamate was removed at the indicated time after the glutamate addition and cell viability was determined 24 h later. For other samples, 10 μM flavonoids were added at the indicated time after the glutamate addition. In all cases cell viability was assessed by the MTT assay 24 h after the glutamate addition. The following flavonoids were used: flavonol (△), 6-HF (□), baicalein (▽), and quercetin (◇).

while kaempferol and anthocyanidin have marginal effects. Other flavonoids such as flavonol, catechin, and chrysin are ineffective against the H_2O_2 toxicity. From the above results, it can be concluded that many flavonoids protect HT-22 cells from several different forms of oxidative stress.

Time course of protection

Because oxidative glutamate toxicity initiates a well-defined temporal cascade of obligatory and sequential events, the mechanism of protection by a compound may be deciphered by the latest time when the compound is still protective after the addition of glutamate [30,38]. Therefore, flavonoids were added to HT-22 cells at 2 h intervals following glutamate, and the cells were then incubated for a total of 24 h. The MTT assay in Fig. 4 shows that the withdrawal of glutamate before 8 h into the cascade prevents subsequent cell death, but the cells cannot reverse the death program after 8 h ("the point of no return"). Effective flavonoids protect cells as late as 10 h after the glutamate addition. These results suggest a latent action by flavonoids. To further determine the protective mechanism by flavonoids, cellular metabolic markers—GSH levels, ROS levels, and Ca^{2+} influx—were examined.

Glutathione levels

Glutamate inhibits cystine uptake, causing the total loss of the cellular GSH within 8 h [42,43]. Cells can be

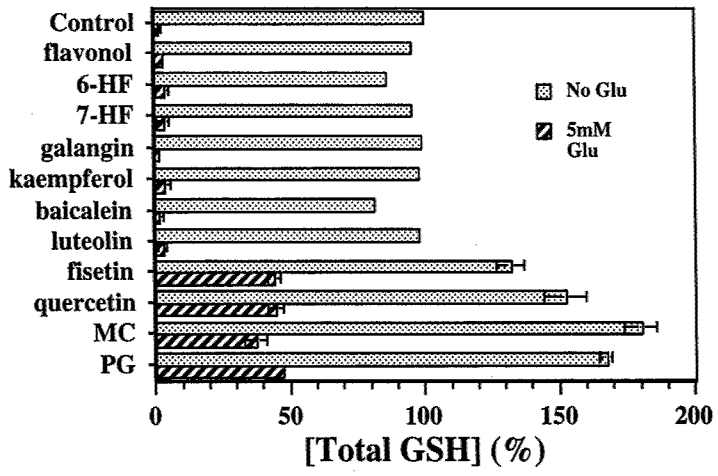

Fig. 5. Effects of flavonoids on cellular levels of glutathione. HT-22 cells were treated with the indicated flavonoids (10 μM) in the absence (dotted bars) or presence (hatched bars) of 5 mM glutamate for 10 h and cellular levels of total glutathione (GSH) were measured as described in Materials and Methods. The GSH level of the control sample (56 ± 1.7 nmol/mg protein) was taken as 100%. The results are the means of triplicate determinations ± SEM from two to three independent experiments.

rescued from glutamate toxicity by mechanisms either dependent upon or independent of GSH metabolism. For example, antioxidants such as vitamin E protect neuronal cells from oxidative glutamate toxicity without affecting the intracellular GSH levels [33,38]. In contrast, dihydroxyphenylglycine, an agonist of Group I metabotropic glutamate receptors, protects neurons by upregulating GSH [49]. To determine if the protection by flavonoids is via GSH metabolism, HT-22 cells were treated with 5 mM glutamate in the presence or absence of a flavonoid for 10 h, harvested, and total GSH determined. As shown in Fig. 5A, some protective flavonoids such as flavonol, galangin, and baicalein do not increase the basal levels of GSH, nor do they prevent GSH loss caused by glutamate. In contrast, quercein and fisetin, as well as PG and MC, increase basal levels of GSH by 30 to 80% relative to untreated cells. Even in the presence of 5 mM glutamate HT-22 cells treated with these compounds maintain 40% to 50% of the GSH level in the untreated cells. It has been shown previously that cells survive glutamate toxicity if the cellular GSH level is more than 15 to 20% of the control level [42,49]. Therefore, quercetin, fisetin, PG, and MC protect HT-22 cells from glutamate toxicity by altering GSH metabolism.

Reactive oxygen species

The loss in cellular GSH up to 85% of the control level causes only a 5- to 10-fold increase in levels of ROS [30]. A greater GSH loss, however, stimulates mitochondria to produce a 100-fold increase in ROS, resulting in cell death [30]. This is clearly shown in the sample treated with 5 mM glutamate and the nonprotective flavonoid chrysin for 11 h (Fig. 6A). A similar analysis was performed for the group of protective flavonoids and the fold increase in the median fluorescence intensity in the presence of 5 mM glutamate with respect to that of the basal level was determined (Fig. 6B). Therefore on the basis of their effect on cellular ROS and GSH, protective flavonoids can be roughly classified into three groups: the quercetin type, the flavonol type, and the galangin type. HT-22 cells treated with glutamate in the presence of the quercetin type (quercetin, fisetin, PG, and MC) accumulate a relatively low level of ROS (Fig. 6B), possibly because these compounds maintain the cellular GSH above the critical level for the explosive generation of ROS (Fig. 5). In contrast, the treatment with the flavonol type (flavonol, 6-HF, and 7-HF) does not prevent the accumulation of ROS in glutamate toxicity. The high level of fluorescence was not due to the autofluorescence of the flavonoids because cells treated only with flavonol did not have a high background (data not shown). Cells treated with the flavonol type also show consistently elevated levels of ROS, even though the cells were at least 90% viable throughout the experiment and for at least 30 h thereafter (Fig. 2A). These results were confirmed by using fluorescent probes dihydrorhodamine 123 or dihydroethidium for ROS detection (data not presented). Finally, the galangin type (galangin, baicalein, kaempferol, and luteolin) show an increase in ROS of less than 10-fold, which is similar to vitamin E, but also have low levels of GSH (Fig. 5) [59].

Antioxidative efficacy

The activity of flavonoids is generally explained by their antioxidative efficacy. But because the flavonol

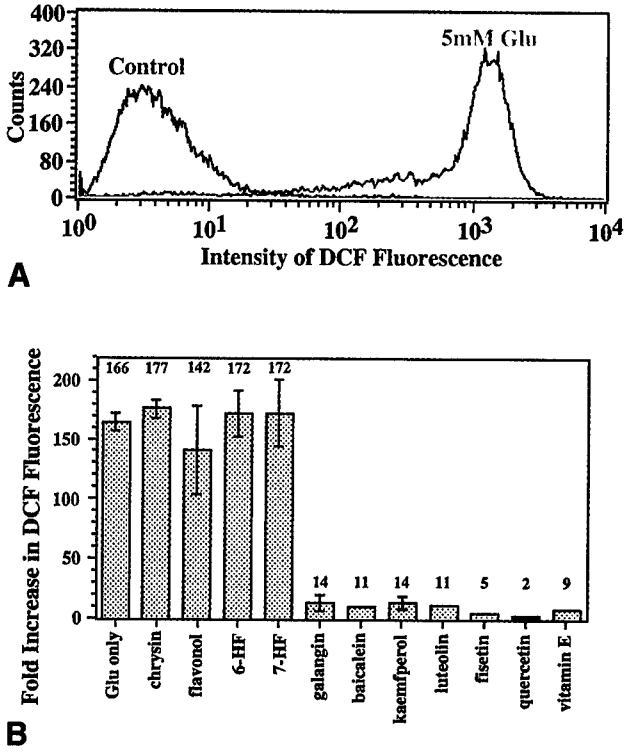

Fig. 6. Effects of flavonoids on ROS levels. HT-22 cells were treated with 5 mM glutamate and chrysin (A) for 11 h and the sample was loaded with H_2DCF-dA and processed as described in Materials and Methods. Levels of ROS were measured with DCF fluorescence and channels of fluorescence intensity were plotted against the counts in each channel. (B) Levels of ROS were measured in the presence of 10 μM flavonoids as above and the fold increase in the median DCF fluorescence intensity with respect to that of the control was plotted. The results are the means of duplicate determinations ± SEM from three independent experiments. Numbers listed above the bar graphs are the levels of the fold increase in DCF fluorescence compared to the control level.

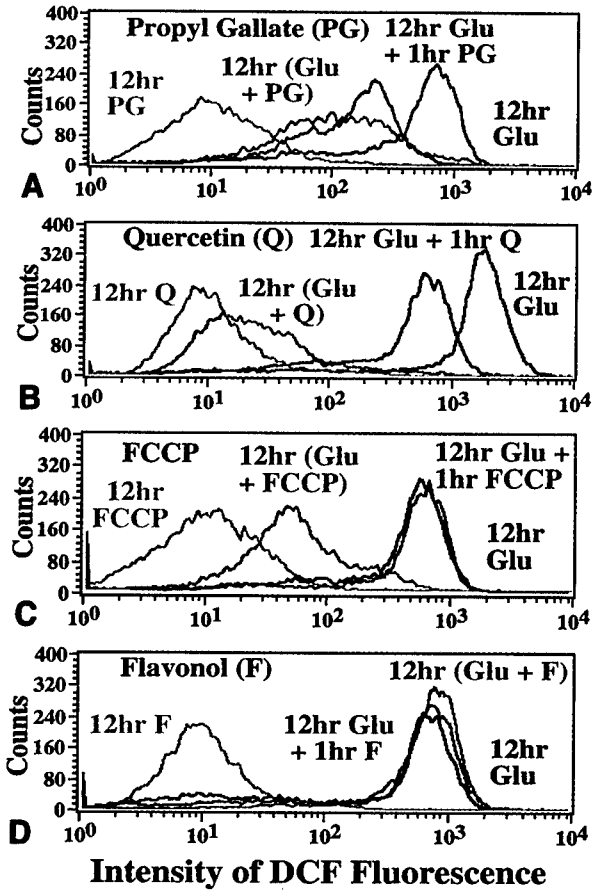

Fig. 7. Antioxidative effects of flavonoids. (A) HT-22 cells were treated either with 10 μM propyl gallate (PG) only (12 h PG − green) or with 5 mM glutamate for 12 h in the absence (12 h Glu − black) or presence of 10 μM PG (12 h (PG + Glu) − blue). In another set of samples, HT-22 cells were treated with 5 mM glutamate for 11 h and 10 μM PG was added for 1 h (12 h Glu + 1 h PG − red). Then, levels of ROS were measured using DCF fluorescence as in Fig. 6 and channels of fluorescence intensity were plotted against the counts in each channel. (B) Same as above except with 10 μM quercetin (Q). (C) Same as above except with 10 μM FCCP. (E) Same as above except with 10 μM flavonol (F).

type (flavonol, 6-HF, and 7-HF) did not prevent the increased accumulation of ROS in HT-22 cells after glutamate treatment, their antioxidative properties were studied using a well-established procedure to determine Trolox Equivalent Activity Concentration (TEAC) [55]. In this procedure, a substance is compared to 1 mM Trolox, a water-soluble vitamin E analog, in its ability to suppress the radical cation of ABTS in an aqueous solution [20]. In addition to TEAC values for some flavonoids reported previously [26], we determined values for several others (Table 1). The TEAC values of many flavonoids do not correlate with the protective efficacy against cellular oxidative stress (Table 1).

The discrepancy between TEAC values and the protective efficacy of flavonoids observed in Table 1 may be attributable to different accessibility of flavonoids to ROS in the two systems. While TEAC values reflect the ability of flavonoids to quench free radicals in aqueous solution, in glutamate toxicity the generation and accumulation of ROS take place in mitochondria and in the cytoplasm. Therefore, to determine the ability of exogenous flavonoids to quench ROS in cells, HT-22 cells were treated with glutamate for 11 h, the time point at which the cells are alive but accumulate high levels of ROS (Fig. 6A; [30]). The flavonoid was then added to the cells in the presence of glutamate for an additional 1 h and processed for the determination of DCF fluorescence as in Fig. 6. HT-22 cells accumulate a high level of ROS after 12 h incubation with 5 mM glutamate (Fig. 7A, "12hr Glu") compared to the control sample treated only with 10 μM PG (Fig. 7A, "12hr PG"). HT-22 cells treated with PG and 5 mM glutamate accumulate a lower level of ROS after 12 h (Fig. 7A, "12hr (Glu + PG)"; see, also Fig. 6B). That PG can also act as an antioxidant is shown by its ability to decrease accumulated levels of ROS within 1 h (Fig. 7A, "12hr Glu + 1hr PG"). Quer-

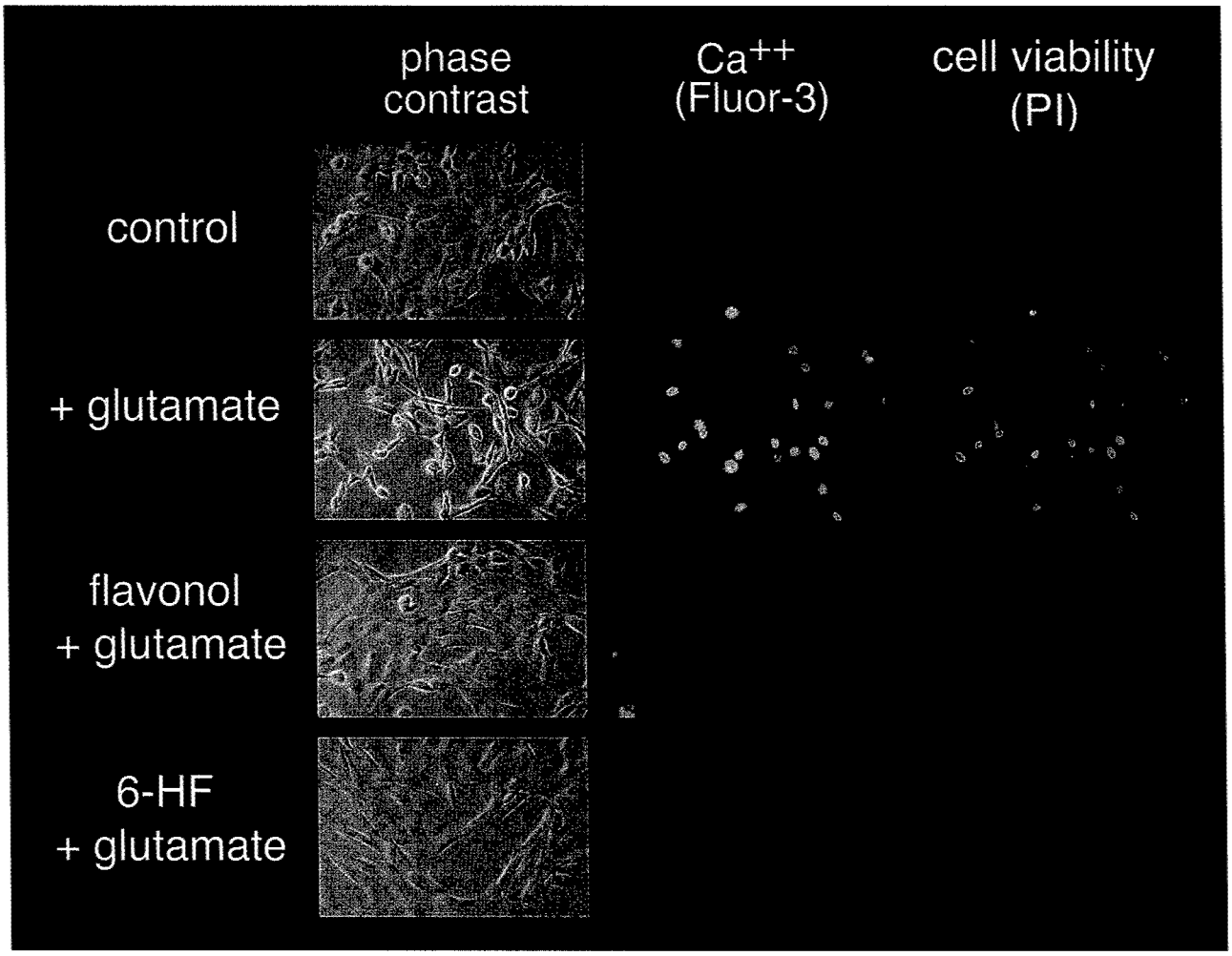

Fig. 8. Effects of flavonoids on Ca^{2+} influx caused by glutamate. HT-22 cells (4×10^5 cells) were seeded in 60 mm tissue culture dishes. 18 h later 5 mM glutamate and indicated flavonoids (10 μM) were added to the cells and incubated for 11 h. Cells were then loaded with 0.5 μM Fluo-3 AM in the presence of 0.005% Pluronic 127 as indicated in Materials and Methods. First column: phase contrast view. Second column: Ca^{2+} levels (Fluo-3 fluorescence). Third column: cell viability (PI fluorescence).

cetin (Fig. 7B) can also quench ROS, indicating the antioxidative property. Other protective flavonoids, baicalein, luteolin, galangin, kaempferol, and fisetin all acted as antioxidants in this system (data not shown).

In contrast, a mitochondrial uncoupler cyanide p-trifluoromethoxyphenylhydrazone (FCCP) decreases levels of ROS and protects HT-22 cells from glutamate toxicity only if added prior to "the point of no return" because mitochondria are the source of ROS in this system (Fig. 7C, "12hr (Glu + FCCP)") [30]. FCCP, however, cannot scavenge ROS if added after the generation of ROS (Fig. 7C, "12hr Glu + 1hr FCCP"). Finally, flavonol does not decrease the accumulated ROS under this condition (Fig. 7D, "12hr Glu + 1hr F"); the ROS level was similar to that in the cells treated with glutamate alone (Fig. 7D, "12hr Glu"). Similar results were obtained with 6-HF or 7-HF (data not shown). These results support the hypothesis that the flavonol type may protect HT-22 cells from oxidative stress not by quenching ROS but by affecting a further downstream step in the cellular metabolism in the glutamate toxicity cascade.

Ca^{2+} influx

In oxidative glutamate toxicity, a 100-fold increase in the intracellular ROS results in the elevation of cytosolic Ca^{2+}, which precedes cell death [30]. Because the flavonol type protects HT-22 cells from glutamate despite high ROS levels (Fig. 6B), it was asked if the surviving cells have elevated levels of cytosolic Ca^{2+}. The ratiometric calcium indicator Indo-1 could not be used due to the autofluorescence of some flavonoids [60]. Therefore, the intracellular Ca^{2+} was monitored with the membrane permeable Ca^{2+} specific fluorescence indicator Fluo-3. Control cells show negligible fluorescence. (Fig. 8; the first row). Cells treated with the flavonol type alone all showed a similar pattern of low intracellular Ca^{2+} (data

not shown). Five mM glutamate treatment for 9 h increases the intracellular Ca^{2+} in HT-22 cells ([30]; Fig. 8, second row), and causes cell death as indicated by PI staining (last column). In the presence of flavonol and 6-HF, the intracellular Ca^{2+} remains low and the cells did not die. Therefore, the flavonoids flavonol and 6-HF protect cells by preventing Ca^{2+} influx despite high intracellular levels of ROS. Similar results were obtained with 7-HF.

DISCUSSION

The above results show that some flavonoids protect neuronal cells from oxidative glutamate toxicity and other forms of oxidative injuries caused by cystine deprivation, BSO, hypoglycemia, and H_2O_2. Using glutamate toxicity as a well-characterized model of oxidative injury, we determined the structural requirements of flavonoids for efficacy of protection. Finally, flavonoids were shown to protect from oxidative stress by three distinct mechanisms: directly affecting GSH metabolism, acting as antioxidants, and maintaining low Ca^{2+} levels despite high levels of ROS.

Structural requirements for protection

Because flavonoids protect neuronal cells by three distinct mechanisms, it is difficult to derive at a single structure-activity relationship for different flavonoids. Nevertheless, three structural determinants required for protection can be deduced from Table 1: the presence of the hydroxyl group on the C3 position, an unsaturated C ring, and hydrophobicity. The requirement of the identical determinants has been described previously in aqueous and lipophilic cell-free systems [7,21,26,61] (see below).

Hydroxyl groups

On the A and C rings, the efficacy of protection is altered most dramatically by the hydroxyl group on the C3 position (see Fig. 1). Thus, flavonol is the only effective monohydroxyflavone. Furthermore, the C3 hydroxyl group converts the ineffective 6- or 7-hydroxyflavone to the protective dihydroxyflavones (EC_{50} = 6 μM). The conversion is attributable not to the number of the hydroxyl groups but to the hydroxyl placement on the C3, because chrysin with hydroxyl groups on C5 and C7 is totally ineffective. The same requirement may also affect the isoflavone genistein, which lacks the C3 hydroxyl group and is thus ineffective in protection. The C3 hydroxyl group cannot be methoxylated as evidenced by the ineffectiveness of 3-methoxyflavone. The importance of the C3 hydroxyl group is also observed in the antioxidant and free radical scavenging activities of flavonoids in the cell-free systems [21,26]. One subtle but significant difference between the two systems is that in the cell-free systems the C3 and C5 positions are considered to be identical in terms of charge dispersion, but their protective efficacy against glutamate is distinct [21]. Thus, chrysin (5,7-dihydroxyflavone) is an effective antioxidant (the TEAC value of 2.52), but an ineffective protectant against glutamate toxicity (Table 1). In contrast, 7-HF (3,7-dihydroxyflavone) is a poorer antioxidant (the TEAC value of 1.65) but a better protectant than chrysin. The difference may be attributable, among other factors, to the involvement of several mechanisms of protection, some of which do not depend on the antioxidant activity (see below).

Two exceptions to the requirement of the hydroxylated C3 are baicalein (5,6,7-trihydroxyflavone) and luteolin (3′,4′,5,7-tetrahydroxyflavone), both of which are protective. These exceptions may arise from the fact that they protect HT-22 cells from glutamate by inhibiting lipoxygenases, an enzyme involved in glutamate toxicity [41], although both of these flavonoids can act as antioxidants (Table 1; Figs. 6 and 7; see below).

On the B ring, the efficacy of protection is influenced only by the hydroxyl groups on both C3′ and C4′ positions (together they form the catechol o-dihydroxy structure). For example, flavonoids with the catechol structure are more protective than the C4′ position alone as seen in luteolin (compared to apigenin) and quercetin (compared to kaempferol) or the dihydroxyl groups on the C2′ and C4′ positions as in morin. The catechol structure is known to give excellent radical scavenging properties [21,26]. Hydroxyl groups on other positions on the B ring have no effects. For example, apigenin (4′,5,7-trihydroxyflavone) is as ineffective in protection, and chrysin (5,7-dihydroxyflavone) and both galangin (3,5,7-trihydroxyflavone) and kaempferol (3,4′,5,7-tetrahydroxyflavone) have a similar EC_{50}. Also, the requirement of these hydroxyl groups may be attributable to the inhibition of lipoxygenases as seen in analogs of flavonoids (chalcones) which inhibit 5-lipoxygenase when the hydroxyl groups are present in comparable positions [62].

Unsaturation

The unsaturation of the C ring in a flavonoid is essential for the protection from glutamate toxicity. This conclusion is based on the comparison of quercetin, taxifolin, and catechin, all of which contain five hydroxyl groups on the identical positions (3,3′,4′,5,7) (see Table 1 and Fig. 1). Quercetin with the unsaturated C ring is the only protective flavonoid of the three, and flavanones

(taxifolin and naringenin) and flavanols (catechin and epicatechin) are totally ineffective (Table 1). The exception to this rule is anthocyanidin, but this can be explained by the decreased hydrophobicity of this compound compared to other flavonoids as discussed below. The unsaturation of the C ring, which allows the electron delocalization across the molecule for the stabilization of the free radical, is also an important factor for antioxidants in the cell-free systems [21,26].

Hydrophobicity

In general, the more hydrophobic a flavonoid is, the more protective it is against glutamate toxicity. Thus, the decrease in hydrophobicity by glycosylation (for example, rutin), polyhydroxylation (myricetin), or ionization (cyanidin) inactivates corresponding protective flavonoids. The placement of the hydroxyl group on the C2′ also decreases hydrophobicity because it is ionized readily [63], resulting in decreased efficacy as seen in morin. The same requirement for high hydrophobicity can be further extended to other antioxidants and free radical scavengers. For example, the derivatization of vitamin E, MC, and PG to decrease hydrophobicity results in the decreased efficacy of protection against glutamate (Table 1). The fact that the spin trap N-tert-butyl-α-phenylnitrone (PBN) is not protective against glutamate may be also attributable to its charge. A hydrophobic antioxidant may easily enter the cytoplasm where ROS are generated and accumulate in glutamate toxicity. Hydrophobicity is also an important determinant of protective compounds in other forms of oxidative stress [64].

Because of the importance of hydrophobicity, TEAC values as defined by Rice-Evans [26] do not predict the efficacy of protection in glutamate toxicity. For example, cyanidin has the TEAC value higher than all other flavonoids except quercetin, but it is totally ineffective in protection against glutamate (Table 1). Also, vitamin E is the most effective of the vitamin E analogs in this system, while vitamin E and Trolox have similar TEAC values (Table 1). Structure-activity relationships of flavonoids from other studies in lipid peroxidation and lipophilicity [65], LDL oxidation [25], lipid peroxidation in membranes [24], inhibition of mitochondrial function [61], and metal chelation [66] also fail to predict their efficacy of protection in the cell-based glutamate toxicity assay. This failure may be attributable to the presence of both hydrophilic and hydrophobic compartments in cells such as the cytoplasm, the plasma membrane, and mitochondria. Therefore, oxidative glutamate toxicity may be a useful screening system to assess biological efficacy of compounds that may protect neurons and other cell types from oxidative stress.

Table 2. The Classification of Flavonoids by Protective Mechanisms

Protection type	Flavonoids and phenolic compounds	GSH metabolism	ROS Scavenger	Anti-Ca^{2+} influx
Flavonol	flavonol	−	−	+
	6-HF	−	−	+
	7-HF	−	−	+
Galangin	galangin	−	+	
	baicelein	−	+	
	kaempferol	−	+	
	luteolin	−	+	
Quercetin	quercetin	+	+	
	fisetin	+	+	
	vitamin E	−	+	
	methyl caffeate	+	+	
	propyl gallate	+	+	

The classification of flavonoids according to protective mechanisms is presented. Protective flavonoids were categorized into three types (flavonol, galangin, and quercetin) depending on the earliest cellular markers affected by the flavonoids in the glutamate toxicity cascade. Three other phenolic compounds are also presented for comparative purposes.

Mechanisms of protection

When HT-22 cells and primary cortical neurons lacking ionotropic glutamate receptors are exposed to glutamate the cells die via a programmed cell death pathway which involves, sequentially, a decrease in intracellular GSH, new protein synthesis, caspase activation, ROS production, LOX activation, quanylate cyclase activation, and finally, the influx of Ca^{2+} via a cGMP gated Ca^{2+} channel [30,40,41,43]. Flavonoids protect HT-22 cells from oxidative glutamate toxicity by interrupting the cell death cascade at three distinct steps; preventing the GSH decrease, blocking ROS production, and inhibiting Ca^{2+} influx (Table 2). Quercetin and fisetin increase GSH levels in HT-22 cells both in the presence and absence of glutamate (Fig. 5). Because glutamate decreases the intracellular level of GSH by inhibiting the uptake of cystine necessary for GSH production [33], the increase in GSH metabolism affords protection from glutamate. Such an increase in the basal level of GSH is often caused by the up-regulation of the rate-limiting enzyme for GSH metabolism, γ-GCS (Fig. 5B) [42,67]. A similar protective mechanism is observed with PG, MG (Fig. 5), and the agonist of metabotropic glutamate receptors DHPG [49]. It has been shown previously that the upstream region of the γ-GCS catalytic gene contains an electrophile responsive element [68] and that compounds such as β-naphthoflavone upregulate the regulatory subunit of γ-GCS [69].

Unlike the quercetin related compounds, which affect GSH metabolism, the galangin group protects HT-22 cells as antioxidants (Table 2). This conclusion is supported by five pieces of evidence. First, these compounds do not affect GSH levels (Fig. 5). Second, like vitamin E,

they maintain levels of glutamate-induced ROS within 2- to 10-fold of the control level (Fig. 6B; [59]). Third, these compounds are able to reduce levels of ROS that have been already accumulated in the cells (Fig. 7). Fourth, the TEAC values of these compounds indicate their antioxidative activities in a cell-free system (Table 1). Fifth, they also protect HT-22 cells from H_2O_2 (Fig. 3B). Because the galangin antioxidants can protect the cells despite the drop in intracellular GSH, GSH is acting as an endogenous antioxidant in the cell death cascade induced by glutamate [30,33].

The third mechanism of protection was observed with the flavonol type compounds (flavonol, 6-HF and 7-HF) (Table 2). These flavonols do not affect GSH metabolism (Fig. 5) nor do they act as antioxidants (Fig. 3B; Fig. 6B, Fig. 7; Table 1). Indeed, cells treated with the flavonols survive (Fig. 2) but accumulate ROS levels indistinguishable from those in cells dying from glutamate (Fig. 6B). Under normal conditions, the Ca^{2+} influx increases and cells die [30]. Despite the accumulation of high concentrations of ROS, the cells treated with the flavonols are able to survive with low levels of cytoplasmic Ca^{2+} (Fig. 8). The only protective compound that shows similar conditions of the metabolic markers is a nonspecific Ca^{2+} channel blocker Co^{2+} [30,38]. Co^{2+} blocks Ca^{2+} influx, resulting in the protection of the cells despite high ROS levels [30]. In a manner similar to Co^{2+}, these three flavonols may maintain low intracellular Ca^{2+} in spite of high levels of ROS. This conclusion is supported by the latent action of the flavonols in the time course experiment: the flavonols protect the cells even when added 10 h after glutamate (Fig. 4). The flavonols may interact directly with a Ca^{2+} channel that is responsible for the final demise of the cell [40], and prevent its opening. Alternatively, they may prevent the signaling mechanism between high ROS levels and the opening of the Ca^{2+} channel. We are currently investigating these possibilities.

It has not been formally ruled out that some of the flavonoids modify glutamate uptake, but this is not the mechanism used to protect the cells. For example, flavonol and galangin do not eliminate the loss of GSH caused by glutamate, showing that glutamate is still present in the medium. Quercetin and all other flavonoids are also protective in toxicity caused by BSO and cystine depletion, mechanisms independent of exogenous glutamate. Finally, the galangin group function directly as antioxidants.

As outlined above, individual flavonoids can protect HT-22 cells from oxidative stress via several different mechanisms (Table 2). Quercetin and fisetin, for example, alter GSH metabolism and act as antioxidants at the same time (Fig. 3B; Fig. 7; Table 1; data not shown). Baicalein and luteolin can act as lipoxygenase inhibitors [41], but they can also act as antioxidants (Table 1, Fig. 3B, Fig. 7). The latency of the efficacy of these flavonoids, as shown in Fig. 4, supports the importance of their action as antioxidants. Finally, some flavonoids may protect the cells from glutamate by directly inhibiting ROS production by mitochondria [61].

In summary, we have identified three protective mechanisms for flavonoids in a cell culture model of oxidative stress. The protective efficacy of flavonoids has also been shown in many animal models of oxidative stress. For example, red wine as well as constituent flavonoids such as quercetin reduce the progression of atherosclerosis in mice deficient in apolipoprotein E [70]. Because cellular oxidative stress is an important factor in various diseases, including arteriosclerosis, ischemia, trauma, Alzheimer's disease, Parkinson's disease, and AIDS as well as aging itself [27,71], flavonoids and flavonoid-containing foods may have multiple beneficial effects in the treatment of these conditions.

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ABBREVIATIONS

GSH—glutathione
ROS—reactive oxygen species
BSO—buthionine sulfoximine
H_2O_2—hydrogen peroxide
HCA—homocysteic acid
FBS—fetal bovine serum
DMEM—Dulbecco's modified Eagle's medium
MTT—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS—phosphate-buffered saline
BSA—bovine serum albumin
SDS—sodium dodecyl sulfate
DCF—dichlorofluorescein
H_2DCF-dA—2′,7′-dichlorodihydrofluorescein diacetate
PI—propidium iodide
TEAC—Trolox equivalent activity concentration
ABTS—2,2′-azinobis(3-ehtylbenzothiazoline 6-sulfonate
6-HF—6-hydroxyflavonol
7-HF—7-hydroxyflavonol
γ-GCS—γ-glutamylcysteine synthetase
PG—propyl gallate
MC—methyl caffeate
DHPG—dihydrophenylglycine
FCCP—cyanide p-trifluoromethoxyphenylhydrazone
PBN—N-tert-butyl-α-phenylnitrone

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Oxytosis: A Novel Form of Programmed Cell Death

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Abstract: Extensive nerve cell death occurs during the development of the central nervous system as well as in episodes of trauma and in neurodegenerative disease. The mechanistic details of how these cells die are poorly understood. Here we describe a unique oxidative stress-induced programmed cell death pathway called oxytosis, and outline pharmacological approaches which interfere with its execution. Oxidative glutamate toxicity, in which exogenous glutamate inhibits cystine uptake through the cystine/glutamate antiporter leading to a depletion of glutathione, is used as an example of oxytosis. It is shown that there is a sequential requirement for de novo macromolecular synthesis, lipoxygenase activation, reactive oxygen species production, and the opening of cGMP-gated channels which allow the influx of extracellular calcium. The translation initiation factor eIF2α plays a central role in this pathway by regulating the levels of glutathione. Finally, examples are given in which the reduction in glutathione, the production of reactive oxygen species, and calcium influx can be experimentally manipulated to prevent cell death. Data are reviewed which suggest that oxytosis may be involved in nerve cell death associated with nervous system trauma and disease.

INTRODUCTION

During episodes of trauma, ischemia, and in many neurodegenerative diseases, the nerve cell faces a situation where it is unable to deal with a fundamental imbalance in the metabolism of oxygen derivatives. This imbalance is caused by a deregulation of electron transport, the activation of certain enzymes, and/or the loss of antioxidants. It results in the accumulation of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet -}$), hydroperoxy radicals ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). ROS can, in turn, directly damage cellular components through the formation of adducts, the destruction of unsaturated C-C bonds, and the oxidation of disulfides. These interactions, as well as the ability of ROS to act as signaling molecules [1], lead to a physiological state called oxidative stress, which frequently results in the death of cells. Because oxidative stress, experimentally identified by the oxidation of lipids, proteins and DNA, is frequently associated with neuropathological conditions, it is important to understand its molecular basis so that therapeutics based upon this information can be designed. This review focuses on a unique form of programmed nerve cell death caused by oxidative stress, which we call oxytosis.

This glutamate-induced cell death pathway was initially identified by Murphy and his colleagues and later termed oxidative glutamate toxicity [2, 3]. However, since its initial description, this pathway has been associated with nerve cell death caused by agents other than glutamate, and therefore warrants a less specific name. We therefore propose the name oxytosis and outline its key features below. We will show that oxytosis is quite distinct from classical apoptotic pathways, and argue that it is likely to be involved in many forms of CNS nerve cell degeneration.

Glutamate and Oxidative Stress

Glutamic acid is the most abundant neurotransmitter in the brain, and indeed one of the most abundant free amino acids. Aside from its requirement in protein synthesis, glutamate has evolved into a molecule with complex biological activities and a large number of membrane receptors and transporters. Glutamate acts as a classical neurotransmitter via its interaction with ionotropic [4] and metabotropic [5] glutamate receptors. In addition, high concentrations of extracellular glutamate are toxic to nerve cells. Therefore extracellular glutamate concentrations are kept low by a family of sodium-dependent glutamate transporters found on the surface of both nerve and glial cells [6]. Glutamate-induced cell death pathways in the nervous system can be caused by either excitotoxicity or oxidative glutamate toxicity (glutamate-induced oxytosis). Excitotoxicity is initiated by the activation of NMDA receptors [7, 8], and excessive NMDA receptor activation leads to Ca^{+2} influx through the ion channel. The rapid build-up of intracellular Ca^{+2} is thought to activate a cell death pathway involving the generation of mitochondria-derived ROS [9, 10]. The other glutamate-induced cell death pathway is oxidative glutamate toxicity [2]. In this case, glutamate acts by inhibiting the uptake of the amino acid,

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cystine, which enters the cell through a cystine/glutamate antiporter [11]. Cystine is one of the three amino acids in glutathione (GSH, γ-glu-cys-gly), the major intracellular reducing agent and antioxidant. When GSH is depleted by glutamate or other conditions by more than 80% for a period of a few hours, cells die by a form of programmed cell death. This cell death pathway and those related to it are the subject of this review. Most of the review will cover oxidative glutamate toxicity, and it will be argued that this is a special case of oxytosis.

Oxidative Glutamate Toxicity

The cystine/glutamate antiporter (x_c^-) is a covalent dimer of a heavy chain (4F2) and a light chain (X_cT) [11]. The light chain is uniquely associated with the (x_c^-) complex, while the heavy chain is found in a variety of amino acid transport systems. Concentrations of extracellular glutamate as low as 100 μM inhibit the import of cystine, functionally depleting the cell of its major antioxidant, GSH [12]. In the case of nerve cells, this usually kills the cells by a process which is inhibited by the antioxidant vitamin E. The physiological relevance of this cell death mechanism has been debated, for it has incorrectly been assumed that in pathological conditions the level of extracellular glutamate does not become sufficiently high to alter cystine transport. However, extracellular glutamate can reach levels exceeding 500 μM in ischemia [13, 14] and over 300 μM in cell culture excitotoxicity paradigms [15, 16]. This high concentration of extracellular glutamate is due to several factors: (1) The concentration of free glutamate and glutamine in the CNS are 5-10 mM and 2-4 mM, respectively [17]. (2) Nerve cells possess an enzyme, glutaminase, which converts extracellular glutamine to glutamate upon cell lysis [15]. (3) Pro-oxidant conditions found in ischemia and trauma shut down the high affinity glutamate transporters on nerve and glia, which normally clear extracellular glutamate [6]. Therefore, under pathological conditions extracellular glutamate in the CNS can reach concentrations sufficiently high to kill nerve cells even if they do not express functional ionotropic glutamate receptors. The question is how do the cells die?

THE CELL DEATH PATHWAY

Glutathione

Figure 1 outlines schematically what is known about the programmed cell death pathway underlying oxidative glutamate toxicity and will serve as a specific example of the more general oxytosis pathway. The majority of the initial observations in our laboratories on glutamate-induced oxidative stress were made with the mouse hippocampal cell line called HT22, and confirmed with newly plated cortical neurons which lack ionotropic glutamate receptors. Many of these observations have been confirmed in other nerve cell lines, including PC12 cells (e.g. [18]). HT22 cells are transformed neuronal cells which express no functional ionotropic glutamate receptors and are therefore not susceptible to excitotoxicity induced cell death [19]. The initiating event in oxidative glutamate toxicity is the loss of GSH from the cells which is caused by the inhibition of cystine uptake by glutamate. While some cells can synthesize cysteine from cystathionine by the enzyme cystathionine-γ-lyase, nerve cells generally do not express this enzyme and are therefore dependent upon extracellular cystine. When cystine uptake is blocked, GSH pools are depleted much faster than protein synthesis is inhibited. In

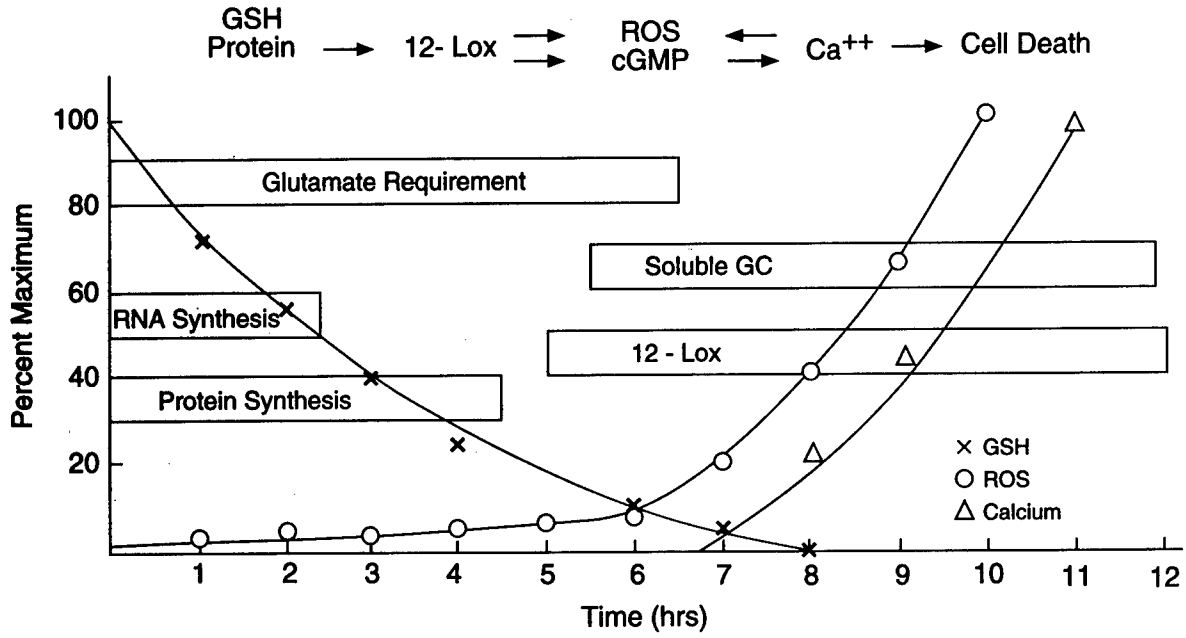

Fig. (1). Schematic diagram of the time course of glutamate-induced cell death in rodent cortical neurons and the HT22 hippocampal nerve cell line. The lines indicate the relative increase or decrease in the molecules indicated and the rectangles indicate the times following the addition of exogenous glutamate when the indicated processes (e.g. RNA synthesis) occur.

fact, there is very little reduction in the rate of protein synthesis up to 4 hr following the inhibition of cystine uptake by glutamate, a time at which GSH levels approach zero [20]. Indeed, *de novo* mRNA and protein synthesis are required, for the inhibition of macromolecular synthesis protects cells from oxidative glutamate toxicity [20, 21]. Other conditions which lead to oxidative stress, such as exposure to peroxides, and cystine deprivation also lead to the reduction of GSH. If cells are exposed to glutamate for 7 hr or longer, the death pathway is irreversible, while for shorter times the cells recover if glutamate is removed.

Reactive Oxygen Species

The exposure of HT22 cells to glutamate leads to a biphasic increase in ROS production (Fig. 1). Between 0-6 hr after the addition of glutamate there is a linear increase in ROS production to about 10% of its maximum value. After 6 hr there is an exponential increase in ROS accumulation to 100- to 200-fold the value in untreated cells. While the early increase in ROS appears to be tightly coupled to the loss of GSH, the later exponential increase does not. When GSH is depleted by L-buthionine-[S,R] sulfoximine (BSO), an irreversible inhibitor of γ-glutamyl-cysteine synthetase (γGCS), the rate limiting enzyme in GSH synthesis, the early phase of ROS production is indistinguishable from that of cells treated with glutamate, while the ROS level in cells treated with 50 μM BSO at 10 hr is only 20% of that in glutamate-treated cells [20]. It is therefore likely that while GSH depletion is necessary for the late exponential increase in ROS production, it is not sufficient. 50 μM BSO does, however, kill the cells in a manner that is similar to oxidative glutamate toxicity.

Lipoxygenase

The decrease in intracellular GSH is tightly coupled to the activation of 12-lipoxygenase (12-LOX). LOXs are dioxygenases that incorporate molecular oxygen into specific positions of polyunsaturated fatty acids, and based upon their site of insertion, are classified as 5-, 12- or 15-LOXs. In the CNS, 12-LOX predominates and produces 12- and 15-hydroxyeicosatetraenoic acid (12- or 15-HETE). The HETES are, in turn, potent activators of soluble guanylate cyclase (sGC). A unique characteristic of 12-LOX is that its enzymatic activity is induced by low GSH levels [22]. Since oxidative glutamate toxicity leads to a decrease in intracellular GSH to values approaching zero, it was asked if this process activates LOX and whether or not LOX activity was required for cell death. Experiments with HT22 hippocampal neurons and cortical primary cultures showed that glutamate activates LOX to produce 12-HETE, and that inhibitors of LOX block glutamate toxicity [23]. Both the general LOX inhibitor nordihydroguaiaretic acid (NDGA) and the 12-LOX inhibitors baicalein (5,6,7-trihydroxyflavone), and cinnamyl-3,4-dihydroxy α-cyanocinnamide completely block glutamate toxicity, while 5-LOX inhibitors are ineffective [23]. When the enzymatic activity of 12-LOX was assayed, there was a large increase which occurred at 6 hr after glutamate addition, the time point at which intracellular GSH approaches zero. BSO-induced cell death is also blocked by LOX inhibitors. Although LOX activity is not required for the loss of GSH, it is required for the exponential increase in peroxide accumulation which occurs at 6 hr post-glutamate (Fig. 1). Therefore LOX activation precedes the second phase of ROS production and is necessary for it to occur. (Fig. 1). However, LOX activation alone is not sufficient because high levels of ROS are not observed with BSO.

Cyclic GMP

The metabolites of 12-LOX have a large number of biological activities, one of which is the activation of soluble guanylyl cyclase (sGC). sGC produces cGMP, which in turn has a vast repertoire of biological effects. As with 12-LOX, the use of specific inhibitors of sGC, such as LY83583, showed that sGC activity is required for glutamate- as well as BSO-induced cell death to occur [24]. The level of intracellular cGMP increases 8 hr after the addition of glutamate, and continues to do so until the cells start to die around 12 hr. sGC inhibitors do not inhibit the depletion of GSH, but do partially block the increase in ROS. Similarly, when cell-permeable analogues of cGMP are added to cells, they either cause cell death or potentiate glutamate-induced cell death [24, 25].

Mitochondrial ROS Production

The production of ROS is central to most forms of programmed cell death (see [26] and references therein). There are many sources of ROS in cells, including mitochondria and a wide array of enzymes including the monoamine oxidases, tyrosine hydroxylase, L-amino oxidase, the lipoxygenases and cyclo-oxygenases, and xanthine oxidase [27]. In the case of oxidative glutamate toxicity, the source of the late exponential burst of ROS at 6 hr post glutamate (Fig. 1) is most likely a FMN group within mitochondrial complex I. Mitochondria are central to ROS production and cell death. The mitochondrial electron transport uncoupler FCCP dissipates the mitochondrial membrane potential [28], blocks the second, exponential, phase of ROS production, but not the first [20], and prevents cell death. Diphenyliodonium (DPI) and clorgyline also block oxidative glutamate toxicity [19]. Because DPI and clorgyline both bind to flavin-containing proteins such as those found in complex I, and since the mitochondria-specific uncoupler FCCP also blocks ROS production and toxicity, it follows that the majority of the late phase ROS production is from mitochondria. Inhibitors of both RNA and protein synthesis also block ROS production and cell death [20, 21]. The source of the initial ROS generation, which may be involved in a signaling pathway, is unknown [1].

Calcium

Calcium entry into cells is a necessary step in cell death caused by oxidative glutamate toxicity. Death is prevented if exogenous Ca^{+2} is removed and the Ca^{+2} channel blocker cobalt prevents glutamate toxicity [2, 29]. Glutamate

induces a large increase in intracellular Ca^{+2} [20, 23]. Following the addition of glutamate there is a 30-50 fold increase in intracellular Ca^{+2} which roughly parallels the increase in ROS, but with a 30-60 min delay (Fig. 1). If the entry of calcium is prevented late in the cell death program, then cell death is inhibited even though the cells have accumulated large amounts of ROS [23]. Therefore increased intracellular ROS is not sufficient to outright kill cells as is frequently assumed. There is, however, an as yet undefined interaction between ROS production, intracellular Ca^{+2} and the influx of extracellular Ca^{+2}, for if cobalt is added earlier in the program before the increase in ROS, there is no late phase increase in ROS [20]. In addition, when mitochondrial ROS production is blocked by FCCP, there is no increase in intracellular Ca^{+2}, nor do the cells die [20]. Therefore Ca^{+2} influx and mitochondrial ROS production are tightly coupled. Since ruthenium red, which is a potent inhibitor of the mitochondrial Ca^{+2} uptake uniporter, blocks late phase ROS production and cell death, it is likely that mitochondrial Ca^{+2} influx is necessary for maximum ROS production [20].

Finally, Ca^{+2} influx is also blocked by inhibitors of sGC [24] and is stimulated by cGMP but not cAMP [25]. It is therefore very likely that Ca^{+2} entry is occurring through a cGMP-gated Ca^{+2} channel. These types of channels have not been well characterized in the brain, but they are central players in the photo-transduction process of neural retina photoreceptor cells. Their possible role in other forms of programmed cell death needs to be more closely examined.

Factors not Involved

A number of parameters which are generally associated with the cell death mediated by classical apoptotic pathways are not required to execute the cell death program initiated by high concentrations of extracellular glutamate and other forms of oxidative stress (Table 1). These include the pro-apoptotic gene Bax, caspase 3 activation, mitochondrial membrane depolarization, DNA fragmentation and cytochrome C release from mitochondria. The oxidative stress pathway can be completed in Bax knockout mice [31] and in the presence of caspase 3 inhibitors [21]. In addition, during oxidative glutamate toxicity, the mitochondria become hyperpolarized as opposed to depolarized (unpublished). Nitric oxide is thought to mediate many forms of cell death, including those associated with CNS ischemia and trauma, but nitric oxide synthase inhibitors fail to block glutamate toxicity [24]. Finally, the morphological characteristics of cells undergoing glutamate-induced cell death are quite distinct from those seen in classical apoptosis.

Table 1. Factors not Involved in Oxytosis

Factor	Reference
Bax	[31]
DNA fragmentation	[21]
Nitric oxide	[24]
Caspase 3 activation	[21]
Nuclear condensation	[21]
Cytochrome C release	Unpublished
Mitochondria membrane depolarization	Unpublished

Morphology

Apoptosis was initially defined as a mechanism of cell death which is "an active, inherently programmed phenomenon" [32]. In tissues, the morphological hallmarks of apoptosis are cell shrinkage, nuclear fragmentation and chromatin condensation. While oxidative glutamate toxicity has some of the features of apoptosis such as the requirement for macromolecular synthesis, it is missing others such as nuclear and DNA fragmentation and chromatin condensation. Since the initial definition of apoptosis was made on the basis of morphology, and since morphology is the only real handle for studying the death of single cells *in vivo*, the morphological events associated with oxidative glutamate toxicity were studied. When examined by electron microscopy, untreated control cells have well-defined intracellular organelles and various patterns of chromatin localization within the nucleus. In contrast, HT22 cells exposed to glutamate for 10 hr suffer severe damage to their endoplasmic reticulum (ER), golgi apparatus and mitochondria. Perhaps the most apparent damage is to mitochondria, which retain both their inner and outer membranes but are very swollen and lose their cristae. The golgi apparatus is also swollen, and contains many vacuoles [21]. Finally, in contrast to apoptotic nuclei, the nuclei of glutamate-treated cells remain intact, with minimal swelling or chromatin condensation. These ultrastructural changes, which occur primarily in the cytoplasm, and are associated with lethal oxidative stress, are quite distinct from those seen during apoptosis. They are, however, very similar to those observed in the developing nervous system [33], as well as in models of ischemia and trauma [34]. In addition, in animals in which two critical caspases, 3 and 9, are genetically deleted, there is a normal amount of developmental cell death, but it occurs by a non-apoptotic pathway which is morphologically indistinguishable from oxidative glutamate toxicity [35]. Thus, these morphological data, in conjunction with the biochemical data, suggest that oxidative stress-induced cell death is quite distinct from that seen in classical apoptosis, and that it is relevant to both pathological and physiological nerve cell death.

Programmed Cell Death and Oxytosis

As outlined above, the turning point for the interest in programmed cell death was the morphological observations of Kerr and colleagues in 1972 [32], followed by the 1980 publication of Wyllie [36], who put the morphological data together with biochemical results showing DNA laddering of genomic DNA. Programmed cell death is a term that is frequently used interchangeably with apoptosis. It was initially used to describe the mechanism by which defined

populations of cells die during development. It was thought that their death is due to a pre-programmed pattern of gene expression which results in cell death at a precise time during development, perhaps based upon an inherent clock or method of counting cell divisions. It is now clear, however, that within the nervous system this type of cell death usually results instead from unsuccessful competition for target tissues and growth factors. Therefore, most developmental forms of cell death are driven by external factors, and the term 'programmed cell death' should be used in a broader sense to apply to cell death mechanisms which require the active participation of the cell in terms of macromolecular synthesis and enzyme activation. In contrast, the term 'necrosis' has been around for over a century and is used to describe a form of cell death characterized by cell swelling (apoptotic cells shrink) and a somewhat selective destruction of cytoplasmic organelles, including mitochondria. Although it is usually assumed that apoptosis and necrosis are unrelated phenomena, recent data has clearly established that the distinction between the two is blurred, and indeed may be nonexistent (see [37] and [1] for more extensive discussion). The case in point is oxidative glutamate toxicity, which by morphological criteria looks very much like necrosis [21], but by biochemical analysis has many of the trappings of apoptosis, including the requirement for macromolecular synthesis. Therefore, the cell actively participates in its own demise, part of Kerr and colleagues' original definition of apoptosis [32].

In addition to oxidative glutamate toxicity, a number of other forms of nerve cell death which have an oxidative stress component appear to follow a very similar cell death program as described above for oxidative glutamate toxicity, for they are inhibited by many of the selective blockers of the oxidative glutamate toxicity pathway. Table 2 shows a comparison of oxidative glutamate toxicity with two more general facilitators of oxidative stress, H_2O_2 and BSO. Most of the blockers of the oxidative glutamate toxicity pathway described above inhibit cell death caused by H_2O_2 and BSO. In addition, nerve cell death caused by glucose starvation and arsenate poisoning is also inhibited by some, but not all, pathway blockers for oxidative glutamate toxicity, showing that there is significant overlap between multiple cell death pathways. The list of mechanistic differences between oxidative glutamate toxicity, as well as H_2O_2 and BSO toxicity, and classical apoptosis, as studied primarily in lymphoid lines, is presented in Table 3. As discussed above, oxidative glutamate toxicity is distinct from classical apoptosis, but it is clearly a form of programmed cell death. Since it appears to be representative of a unique class of cell death programs and is initiated by more than one mechanism, then it should be distinguished by its own name. We propose the name *oxytosis* to reflect the extensive involvement of ROS production and oxidative stress in this distinct cell death pathway.

Table 2. Shared Cell Death Pathways

Inhibitor	Oxidative Glutamate Toxicity	H_2O_2	BSO
Cycloheximide	+	+	+
Vitamin E	+	+	+
Baicalein (LOX)	+	+	+
LY83583 (sGC)	+	+	+
Cobalt (Ca^{++})	+	+	+
Clorgyline (Mito)	+	-	-
DPI (Mito)	+	-	-
Dopamine (D4)	+	+	+

Table 3. Comparison of Apoptosis and Oxytosis

	Apoptosis	Oxytosis
Nuclear fragmentation	+	-
Chromatin condensation	+	-
Cytoplasmic vacuolation	±	+
Mitochondrial swelling	±	+
DNA fragmentation	+	-
Caspase 3 processing/activation	+	-
Protection by specific caspase inhibitors	+	+
RNA and protein synthesis	±	+

PATHWAYS INVOLVED IN PROTECTION FROM OXIDATIVE STRESS-INDUCED NERVE CELL DEATH

We have identified a number of inhibitors of oxidative glutamate toxicity. These inhibitors interrupt the cell death cascade at one of three distinct steps: preventing the GSH decrease, blocking ROS production and inhibiting Ca^{+2} influx. In the following paragraphs we will describe what we know about the mechanisms underlying the inhibition of each of these three steps and indicate areas where further investigation is needed.

Inhibitors of GSH Depletion

Several distinct compounds affect GSH metabolism such that GSH levels are maintained in the presence of glutamate and often increased in its absence as well. These compounds include the group I metabotropic glutamate receptor agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG) [12], methyl caffeate, propyl gallate, the flavonoids, quercetin and fisetin, [30] and thapsigargin [38]. An increase in the level of GSH is often caused by the up-regulation of the rate-limiting enzyme for GSH biosynthesis, γ-GCS. The upstream region of the γ-GCS gene contains an antioxidant response element (ARE) which is known to be activated by some of these compounds [39]. Therefore, at least some of the compounds which increase GSH levels in nerve cells may act on the ARE to increase the transcription and subsequent translation of γ-GCS. In glutamate-resistant HT22 cells, there is a 2.6- to 4-fold increase in the activity of γ-GCS [40]. However,

there is not a good correlation between resistance to glutamate and the steady state level of γ-GCS mRNA, suggesting that other mechanisms are responsible for the increase in γ-GCS activity.

An additional mechanism for the regulation of γ-GCS levels is suggested by our recent work on the role of e-IF2α in the protection of cells from oxidative stress. Using the HT22 cells in combination with an expression cloning strategy, two glutamate-resistant clones were isolated which contain antisense gene fragments of the translation initiation factor eIF2α and express a lower amount of eIF2α than the control cells [41]. The sensitivity of the cells to glutamate is restored when they are transfected with full length eIF2α. These clones are also resistant to other forms of oxidative stress including hydrogen peroxide and tert-butyl peroxide, supporting the idea that there is a shared oxytosis program which is activated in response to distinct stresses. The cells expressing the eIF2α antisense fragment have increased levels of GSH and maintain high GSH concentrations in the presence of glutamate. This increase in GSH correlates with an increase in the level of γ-GCS protein but not mRNA. Therefore, eIF2α regulates γ-GCS activity by a translational, rather than a transcriptional, mechanism.

eIF2α activity is normally regulated by phosphorylation. eIF2α forms part of a trimeric complex involved in the initiation of protein translation. The α subunit dictates whether protein synthesis will or will not take place and is often referred to as the control point for protein synthesis. The eIF2 complex brings the 40S ribosomal subunit together with the initiating $tRNA_{MET}$ when eIF2α is bound to GTP. Upon hydrolysis of GTP to GDP, the complex is no longer active and protein synthesis in not initiated. GDP/GTP exchange requires the activity of the guanine nucleotide exchange factor eIF2B. However, when eIF2α is phosphorylated on serine 51, it sequesters eIF2B, thus inhibiting both the GDP/GTP exchange and protein synthesis. Infection of HT22 cells with a construct expressing the S51D mutant of eIF2α, which acts as a constitutively phosphorylated form of the protein, results in increased resistance to glutamate which correlates with an ability to maintain a high GSH concentration in the presence of glutamate and an increased level of γ-GCS [41]. Furthermore, thapsigargin as well as DHPG, methyl caffeate, propyl gallate and the flavonoids fisetin and quercetin induce eIF2α phosphorylation in HT22 cells which correlates with increased levels of γ-GCS (unpublished results), suggesting a common pathway shared by mulitple protective agents.

There are four known eIF2α kinases: GCN2, HRI, PKR and PERK. The interferon-inducible, double stranded RNA-activated kinase, PKR, phosphorylates eIF2α in response to viral infection while the heme-regulated eIF2α kinase, HRI, coordinates globin synthesis with heme availability in erythroid cells. In contrast, both PKR-like ER kinase (PERK) and general control nonrepressed-2 kinase (GCN2) phosphorylate eIF2α in cells responding to stress such as ER stress (PERK) or amino acid deprivation (GCN2). $PERK^{-/-}$ cells are unable to phosphorylate eIF2α in response to ER stress and show a significant impairment in their ability to survive this stress [42]. PERK also appears to be the kinase responsible for eIF2α phosphorylation in the early post-ischemic brain [43]. However, the role of post-ischemic eIF2α phosphorylation in the subsequent nerve cell death has yet to be resolved.

In spite of the potential negative effect of eIF2α phosphorylation on general protein translation, recent evidence suggests that the activation of both GCN2 and PERK selectively increases the translation of specific proteins including Activating Transcription Factor 4 (ATF4) [44] which, in turn, results in the transcriptional induction of several downstream genes including CHOP (GADD153), BiP [44] and GADD34 [45]. Whether γ-GCS translation is also induced by PERK- and/or GCN-dependent phosphorylation of eIF2α remains to be determined but could underlie the increase in GSH levels induced by DHPG, methyl caffeate, propyl gallate, quercetin, fisetin, and thapsigargin.

Inhibitors of ROS Production

A number of distinct compounds block the exponential increase in ROS which is seen beginning at 6 hr following the addition of glutamate to cells and maintain ROS levels within 2- to 10-fold of the control level. Some of these compounds, such as flavonoids related to galangin, act directly as antioxidants [30]. Thus, these compounds not only have radical scavenging abilities similar to vitamin E but they can also reduce the levels of ROS that have already accumulated in cells treated with glutamate [30].

The exponential increase in ROS production which begins around 6 hr after the addition of glutamate to cells is derived from mitochondria (Fig. 1). Another set of compounds which block oxidative glutamate toxicity act directly on mitochondria to block ROS production. These compounds include the monoamine oxidase-A (MAO-A) inhibitor clorgyline, diphenyleneiodonium (DPI), which is an inhibitor of flavoenzymes such as NADPH oxidase [19] and uncouplers and inhibitors of the electron transport chain such as FCCP and antimycin A, respectively [20]. Not only do all of these compounds inhibit the exponential phase of ROS production but they also block cell death induced by glutamate as well as a number of other compounds which induce oxidative stress. Both MAO-A inhibitors [20] and DPI [46] directly inhibit the production of ROS by mitochondria. Interestingly, none of these compounds could block cell death induced by BSO ([47] and unpublished results). This observation is consistent with the lack of an exponential phase of ROS production in BSO-induced cell death.

A third group of compounds also blocks the exponential phase of ROS production. These compounds are neither antioxidants nor inhibitors of mitochondrial ROS generation. This group includes the phorbol ester, tetradecanoylphorbol acetate (TPA), inhibitors of p38 MAP kinase (p38 MAPK; see below), protein and RNA synthesis inhibitors and the caspase inhibitor, Ac-YVAD-CMK. Treatment of HT22 cells or primary cortical neurons with TPA, an activator of conventional and novel isozymes of protein kinase C, inhibits oxidative glutamate toxicity in a dose dependent manner [29]. Recently, it was found that

TPA acts on multiple intracellular kinases to protect nerve cells from death induced by oxidative stress [48]. TPA treatment has distinct effects on different members of the mitogen activated protein kinase (MAP kinase) family of protein kinases (for review see [49]). These serine-threonine kinases are activated by dual phosphorylation in response to a variety of extracellular stimuli. In mammalian cells at least three distinct members of the MAP kinase family are expressed: ERKs (also known as MAPKs), stress-activated protein kinase (also known as c-Jun NH2-terminal kinase (JNK)) and p38 MAPK. ERKs are activated by growth factors and are primarily involved in cell proliferation and differentiation whereas JNK and p38 are primarily activated in response to various forms of stress and are often implicated in inflammatory responses, cell cycle arrest, DNA repair and cell death. Thus, TPA treatment of HT22 cells results in the rapid activation of ERKs which in turn results in both the inactivation of p38 MAPK and the activation of JNKs. Both the inactivation of p38 MAPK and the activation of JNKs appear to be required for protection since p38 MAPK inhibitors also protect the cells from oxidative stress-induced cell death and transfection of cells with a dominant negative JNK construct reduces TPA-mediated protection. In addition, TPA leads to the rapid downregulation of the novel PKC isozyme, PKCδ, whose activity is associated with cell death in other systems. In particular, PKCδ was shown to interact with mitochondria and this interaction appears to be involved in its ability to promote cell death [50, 51]. Thus, the downregulation of PKCδ by TPA treatment may play a role in the ability of TPA to block the exponential phase of ROS production.

The protective action of TPA as well as p38 MAPK inhibitors also may be related to the protective effects of RNA and protein synthesis inhibitors, for many of the substrates of the MAPK family members are transcription factors [49]. Thus, it is likely that glutamate treatment results in the specific transcription of one or more proteins which promote cell death. Furthermore, the data suggest that not only do these proteins promote cell death, but that they also play critical roles in regulating the exponential increase in ROS production. In addition, ERK activation may have direct effects on ROS production. For instance, the ability of nerve growth factor re-addition to acutely suppress ROS production following NGF withdrawal was directly correlated with ERK activation since a specific inhibitor of ERK activation blocked the NGF-dependent suppression of ROS formation [52]. However, how the different MAPK family members can directly or indirectly affect ROS production remains to be elucidated.

Inhibitors of Ca^{+2} Influx

Several distinct compounds also block Ca^{+2} influx. These include dopamine and several related ligands [25] and the flavonoids, flavonol, 6-hydroxy flavonol and 7-hydroxy flavonol [30]. cGMP-dependent Ca^{+2} channels are opened near the end of the glutamate-induced cell death pathway and play a critical role in the cell death process [24]. Dopamine, as well as the dopamine agonists apomorphine and apocodeine, appear to act after the generation of cGMP because they can also block cell death induced by the cell permeable cGMP analog pCPT-cGMP. Since it was shown that the protective effects of dopamine and related ligands are mediated by D4 receptors, this suggests that the activation of these receptors prevents the opening of cGMP-operated Ca^{+2} channels by an as yet undefined mechanism.

Other Inhibitors

A number of other inhibitors of oxidative glutamate toxicity have been described in the literature. However neither the precise steps at which they act nor the mechanisms underlying their protective effects are known. Micromolar concentrations of estrogens block oxidative glutamate toxicity in the HT22 cells by a mechanism which appears to be independent of estrogen receptors [53] but may involve other nuclear steroid receptors [54]. Indeed, RU486, a potent antagonist of progesterone and glucocorticoid receptors, protects HT22 cells, as well as primary cultures of nerve cells, from oxidative stress-induced cell death [55]. Estrogen at concentrations ranging from 0.1 nM to 50 μM, also protect a variety of other nerve cell lines, as well as primary cultures of nerve cells, from oxidative stress and this protection also does not appear to be mediated solely by a classical estrogen receptor-dependent mechanism [56]. Although it was suggested that estrogens protect cells from oxidative stress by direct antioxidant activity [57], they can also have other effects on cells which may contribute to their neuroprotective effects. In particular, estrogens can activate ERKs and alter Ca^{+2} homeostasis [56]. A further investigation of the effects of estrogens on the different steps of the cell death cascade induced by oxidative stress should help resolve these questions.

Finally, a few other reagents block oxidative glutamate toxicity but again their mode of action has not been studied in detail. Geldanamycin blocks oxidative glutamate toxicity in HT22 cells [58]. This benzoquinoid ansamycin induces the synthesis of heat shock proteins, hsp70 and hsp90. Geldanamycin has no effect on the decrease in GSH, but its effects on ROS production and Ca^{+2} influx were not reported. Induction of hsp70 synthesis is implicated in the protection of nerve cells from various forms of stress including serum withdrawal, ischemia and excitotoxicity [59-61]. However, whether hsp70 synthesis or the synthesis of other heat shock proteins is directly correlated with protection by geldanamycin, or any other compound, remains to be determined. The non-opiate analgesic, flupirtine and the related compound retigabine [62] block oxidative glutamate toxicity in PC12 cells through a mechanism which appears to be independent of their actions as antioxidants. Neither agent was able to block the glutamate-dependent decrease in GSH levels, but further aspects of the cell death pathway were not studied. Melatonin also prevents oxidative stress-induced cell death in both the HT22 cells and rat hippocampal brain slices [63]. Melatonin reduces ROS production, but the mechanism underlying this effect was not determined. Very recently, a series of cyclopentenone prostaglandin derivatives were shown to protect HT22 cells from oxidative glutamate toxicity by an unknown mechanism [64].

CONCLUSIONS

In summary, oxidative stress kills nerve cells by a pathway which is distinct from classical apoptosis. Although nerve cells dying by this pathway display a number of morphological features characteristic of necrosis, the cells clearly play an active role in their demise since cell death is blocked by inhibitors of macromolecular synthesis. Since this pathway represents a cell death program which is not only distinct from both classical apoptosis and necrosis but is also dependent upon oxidative stress and ROS production, we propose for it the name oxytosis. Oxytosis involves at least three distinct steps: a decrease in GSH levels to less than 20% of control values, an increase in the production of ROS and a massive influx of Ca^{+2} which occurs shortly before cell death. Compounds which are capable of inhibiting each of these steps have been described, although in many cases their exact modes of action remain to be determined. Nevertheless, many of these compounds may prove useful in reducing the negative consequences following the exposure of nerve cells to oxidative stress.

ABBREVIATIONS

ARE = Anti-oxidant response element

BSO = L-Buthionine-[S,R]-sulfoximine

DHPG = (R,S)-3,5-Dihydroxyphenylglycine

DPI = Diphenyleneiodonium

eIF2α = Eukaryotic initiation factor 2α

ERK = Extracellular signal regulated kinase

FCCP = Carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone

γ-GCS = γ-Glutamyl cysteine synthetase

GSH = Reduced glutathione

JNK = c-jun N-Terminal kinase

LOX = Lipoxygenase

MAPK = Mitogen activated protein kinase

MAO = Monoamine oxidase

PKC = Protein kinase C

ROS = Reactive oxygen species

sGC = Soluble guanylyl cyclase

TPA = 12-*O*-Tetradecanoylphorbol-13-acetate

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Specificity of resistance to oxidative stress

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Abstract

Two clonal nerve-like cell lines derived from HT22 and PC12 have been selected for resistance to glutamate toxicity and amyloid toxicity, respectively. In the following experiments it was asked if these cell lines show cross-resistance toward amyloid beta peptide (Aβ) and glutamate as well as toward a variety of additional neurotoxins. Conversely, it was determined if inhibitors of oxytosis, a well-defined oxidative stress pathway, also protect cells from the neurotoxins. It is shown that both glutamate and amyloid resistant cells are cross resistant to most of the other toxins or toxic conditions, while inhibitors of oxytosis protect from glutathione and cystine depletion and H_2O_2 toxicity, but not from the toxic effects of nitric oxide, rotenone, arsenite or cisplatin. It is concluded that while there is a great deal of cross-resistance to neurotoxins, the components of the cell death pathway which has been defined for oxytosis are not used by many of the neurotoxins.

Keywords: amyloid, glutamate, oxidative stress, oxytosis, programmed cell death, resistance.

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Oxidative stress, defined in terms of an imbalance between reactive oxygen species (ROS) production and destruction, is thought to be involved in nerve cell death associated with ischemia, trauma and neurodegenerative diseases such as Parkinson's and Alzheimer's. In all of these pathologies a fraction of the nerve cells survive the initial insult, suggesting that protective mechanisms are available to some cells. We have previously isolated a series of cell lines which are resistant to oxidative stress caused by amyloid beta peptide (Aβ) or oxidative glutamate toxicity (Sagara *et al.* 1996, 1998). Here we ask if there is any overlap in the resistance of the Aβ and glutamate resistant cell lines to the original toxic agents as well as to a variety of other neurotoxins. In addition, the commonalities of the cell death pathways used in response to several additional neurotoxins are examined.

There are several ways in which the oxidative burden of cells can be regulated. One of these is through extracellular glutamate. Although glutamate is generally thought of as both a neurotransmitter and an excitotoxin, extracellular glutamate can also kill neurons through a non-receptor mediated pathway which involves the glutamate-cystine antiporter, system Xc^- (Bannai and Kitamura 1980; Murphy *et al.* 1989; Sato *et al.* 1999). Under normal circumstances the concentration of extracellular cystine is high relative to intracellular cystine, and cystine is imported via the Xc^- antiporter in exchange for intracellular glutamate. Cystine is rapidly converted to cysteine and utilized for protein synthesis and to make the antioxidant glutathione (GSH). However, when there is a high concentration of extracellular glutamate, the exchange of glutamate for cystine is inhibited and the cell becomes depleted of cysteine and GSH, resulting in severe oxidative stress. The cell eventually dies via a series of well defined events which have the characteristics of both apoptosis and necrosis, a process called oxytosis (for a review see Tan *et al.* 2002). Oxytosis has been most extensively studied in HT22 cells. HT22 cells are immortalized mouse hippocampal neurons that lack ionotropic glutamate receptors but die within 24 h after exposure to 1–2 mM glutamate. Several HT22 subclones have been selected for growth in high exogenous glutamate and are over 10-fold more resistant to glutamate than the parental cells (Sagara *et al.* 1998). These cell lines have increased expression of catalase, but not glutathione peroxidase (GPx) or superoxide dismutase. In addition, the activities of three enzymes involved in GSH metabolism, γ-glutamylcysteine synthetase, GSH reductase, and GSH-S-transferase, are elevated. Since transfection of some of these activities into the wild-type cell line does not confer complete resistance, it is likely that additional molecules play a role in the resistance pathway.

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Abbreviations used: Aβ, amyloid beta peptide; BSO, buthionine-[*S,R*]-sulfoximine; GPx, glutathione peroxidase; MPTP, 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine; ROS, reactive oxygen species.

As with oxidative glutamate toxicity, reactive oxygen species (ROS) are involved in Aβ-induced cell death (Behl *et al.* 1994). Clones of the rat nerve-like cell line PC12 have been selected that are resistant to exogenously applied Aβ (Sagara *et al.* 1996). In these cells there is increased expression of both catalase and GPx, and the transfection of these enzymes into the wild-type line partially increases Aβ resistance. As with resistance to glutamate toxicity, a number of additional undefined factors are responsible for their overall resistance to amyloid.

As oxidative stress in the CNS caused by glutamate and Aβ may occur concomitantly or sequentially during both the aging process and AD pathology, it is important to determine if cells which survive one insult may be more resistant to the other. To this end the following experiments compare the cross-resistance of Aβ and glutamate resistant cell lines to their respective selective agents as well as to a number of additional toxins. We also examine the overlap in the cell death programs initiated by several additional neurotoxins. It is shown that while there is indeed a shared resistance to a wide spectrum of pro-oxidant conditions, the cells die by distinct programmed cell death pathways initiated by the individual neurotoxins.

Materials and methods

Cell culture and toxicity studies

The HT22 hippocampal nerve cell line is a subclone of HT4 (Morimoto and Koshland 1990), which was selected for its sensitivity to glutamate toxicity. The cells do not possess active ionotropic glutamate receptors and are not subject to excitotoxicity (Davis and Maher 1994). The glutamate resistant cell line (HT22r2) was selected for growth in 10 mM glutamate and is maintained in 2 mM glutamate (Sagara *et al.* 1998). HT22 cells are propagated in Dulbecco's modified Eagle's medium (DMEM) (Vogt and Dulbecco 1963) supplemented with 10% fetal bovine serum. Cell survival was determined by the MTT (3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described (Schubert *et al.* 1992). In HT22 cells the MTT assay correlates with cell death as determined by trypan blue exclusion and a colony forming assay (Davis and Maher 1994). Briefly, HT22 cells are dissociated with pancreatin (Life Technologies, Gaithersburg, MD) and seeded onto 96-well microtiter plates in 10% fetal bovine serum at a density of 2.5×10^3 cells per well in 100 μL medium. The next day cells are treated with various reagents according to the experimental design. Twenty hours after the addition of glutamate, 10 μL of the MTT solution (2.5 mg/mL) is added to each well and the cells are incubated for 4 h at 37°C. Solubilization solution (100 μL: 50% dimethylfomamide, 20% SDS, pH 4.8) is then added to the wells and the next day the absorption values at 570 nm are measured. The results are expressed relative to the controls specified in each experiment, and were subjected to statistical analysis (Student's *t*-test).

Rat pheochromocytoma (PC12) cells are a subclone of a high-passage cell line originally obtained from L. Greene (Greene and Tischler 1976). PC12 cells were grown on tissue culture dishes (Falcon, Indianapolis, IN) in DMEM supplemented with 10% fetal bovine serum and 5% horse serum. Aβ-resistant cells were selected for growth in high concentrations of Aβ and are maintained in the presence of 5 μM $A\beta_{(25-35)}$. The data presented here are from one Aβ-resistant clone, AβrCl 8 (Sagara *et al.* 1996). For amyloid toxicity the MTT assay does not necessarily reflect cell viability although it does measure an early necessary event in the cytotoxicity pathway (Liu and Schubert 1997; Liu *et al.* 1997). Therefore growth curves were also done to measure toxicity.

Materials

The caspase inhibitor, AcYVADfmk, was obtained from Bachem (Torrance, CA, USA) (R,S)-3,5-dihydroxyphenylglycine (DHPG) from Tocris-Cookston (England), and all other reagents were from Sigma (St. Louis, MO, USA).

Results

As both glutamate and Aβ are toxic to nerve cells, it was asked if cells selected for resistance to one of these toxins are more or less resistant to the other. Two cell lines were used. The hippocampal nerve cell line HT22, which is sensitive to glutamate, and the PC12 sympathetic nerve-like line which has been extensively studied with respect to amyloid toxicity. Clonal cell lines that are very resistant to each toxin were selected by growth in the presence of high concentrations of Aβ or glutamate (Sagara *et al.* 1996; Sagara *et al.* 1998). For example, the EC_{50} for killing HT22 cells is 1.5 mM glutamate, while the resistant clone requires 25 mM glutamate (Table 1). Similarly, Aβ slows down the rate of growth of wild type PC12 cells but has no effect on the PC12 resistant clone 8 (Fig. 1a). Likewise, using the MTT assay, which measures the biological activity of the Aβ peptide (Liu and Schubert 1997), the EC_{50} is increased from 10 μM Aβ to 55 μM Aβ (Table 1). When the cells selected for glutamate resistance are screened for Aβ toxicity and *vice versa*, there is a surprising degree of cross resistance relative to their parental cell lines (Fig. 1b, Table 1). The Aβ resistant PC12 line is 8 times more resistant to glutamate than its parental cell line, while the glutamate resistant HT22 clone is about twice as resistant to Aβ as the wild type clone. These data show that selection for resistance to one neurotoxin can lead to resistance to other toxins.

To determine if the cross-resistance can be generalized to other forms of neurotoxicity, the toxicity of a variety of additional neurotoxins or toxic conditions were examined on all four cell lines. Three conditions, cystine depletion, GSH depletion, and H_2O_2 toxicity were chosen because of their relationship to the oxytosis pathway (Tan *et al.* 2002), while five additional toxins (arsenite, cisplatin, MPP +, rotenone, and nitric oxide) were selected because their toxicities are thought to involve reactive oxygen species (ROS) (see later). Although the depletion of intracellular glutathione by buthionine-[*S*,*R*]-sulfoximine (BSO) and cystine depletion

	HT22	HT22 r2	PC12	PC12r8
Aβ	15 ± 3 μM	35 ± 4 μM	10 ± 1 μM	55 ± 7 μM
Glutamate	1.5 ± 0.2 mM	25 ± 2 mM	10 ± 3 mM	87 ± 2 mM
BSO	5 ± 1 μM	200 ± 8 μM	500 ± 30 μM	> 2 mM*
ΔCys	129 ± 9 μM	20.6 ± 2 μM	55 ± 5 μM	2.6 ± 0.5 μM
H_2O_2	20 ± 3 μM	200 ± 9 μM	150 ± 26 μM	400 ± 30 μM
Arsenite	6 ± 1 μM	95 ± 8 μM	35 ± 3 μM	105 ± 11 μM
Cisplatin	78 ± 12 μM	100 ± 22 μM	500 ± 120 μM	500 ± 60 μM
MPP+	1.8 ± 0.3 μM	1.9 ± 0.5 μM	2 ± 0.5 mM	6.5 ± 1.5 mM
Rotenone	3.5 ± 0.2 μM	14 ± 6 μM	3 ± 1 μM	7 ± 2 μM
NO	0.5 ± 0.2 mM	0.4 ± 0.1 mM	0.2 ± 0.1 mM	0.3 ± 0.1 mM

Table 1 Glutamate and Aβ Resistant Lines (EC50)

Exponentially dividing cultures of HT22 and PC12 cells as well as their clones resistant to glutamate and Aβ, respectively, were plated in 96 well plates at 2.5×10^3 per well and 18 h later various concentrations of the indicated toxins were added and the cells incubated for an additional 20 h before the MTT viability assay. In all cases, cells were also visually scored for toxicity and both sets of data were in agreement. The toxins were added in triplicate at 3-fold serial dilutions and the EC_{50} for toxicity is given as the mean of 3 determinations plus or minus the standard error of the mean. ΔCys means cystine depletion. The normal concentration of cystine in the culture medium is 260 μM. *The cells are resistant to BSO up to a concentration of 2 mM.

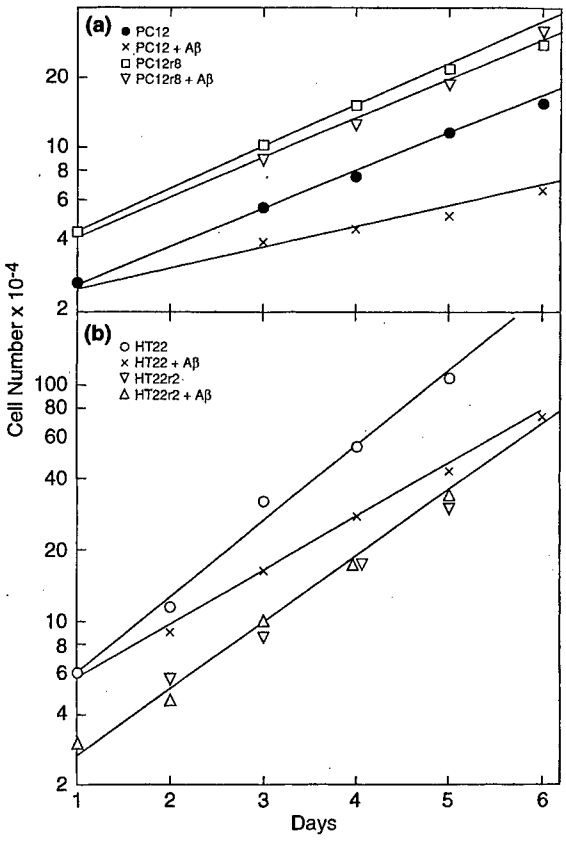

Fig. 1 Aβ is ineffective in slowing the growth rate of both glutamate and Aβ resistant cells. Exponentially dividing cells were placed in 35 mm tissue culture dishes, and the following day (1) 10 μM $A\beta_{25-35}$ was added to some of the cultures. Viable cell counts were done daily following the addition of Aβ. (a), ●-●-●, PC12; x-x-x, PC12 + Aβ; □-□-□, PC12r8; ▽-▽-▽, PC12r8 + Aβ. (b), ○-○-○, HT22; x-x-x, HT22 + Aβ; ▽-▽-▽, HT22r2; Δ-Δ-Δ, HT22r2 + Aβ.

(ΔCys) are related to cell death caused by exogenous glutamate due to the fact that they both deplete GSH, there are mechanistic differences (Tan *et al.* 2002). Both glutamate and Aβ resistant cell lines are, however, much more resistant than their parental cells to both cystine starvation and BSO toxicity (Table 1). In the case of HT22, 50% of the wild-type cells are killed by the reduction of cystine in the culture medium from 260 μM (normal) to 129 μM, while 50% of the resistant cells will survive in 20 μM cystine (Fig. 2). A similar increase in resistance to cystine starvation occurs with PC12r8 (Fig. 2). It should be noted that there are also very large baseline differences between the sensitivities of the

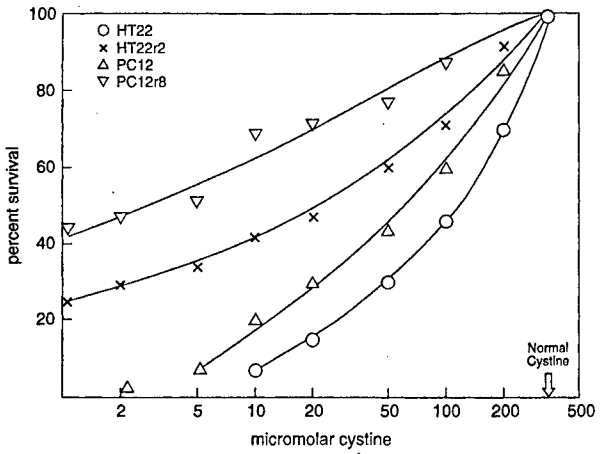

Fig. 2 Glutamate and Aβ resistant cell lines are also resistant to cystine deprivation. Exponentially dividing cultures of the indicated cell line were exposed to the indicated concentrations of cystine. Twenty-four hours later cell viability was determined by the MTT assay and confirmed by visual counting. Δ-Δ, PC12; ▽-▽, PC12r8; ○-○, HT22; x-x, HT22r2.

wild type HT22 and PC12 cell lines to the different toxic conditions. There could be many reasons for these differences but they are not known at this time.

Hydrogen peroxide is frequently used as a standard for the induction of oxidative stress in cultured cells. Since both oxytosis and Aβ toxicity have peroxide intermediates in their cell death pathways (Behl *et al.* 1994; Tan *et al.* 2001), it would be predicted that both resistant cell lines would also be more resistant to H_2O_2. The data in Table 1 show that this is indeed the case. As with glutamate and BSO, it takes a much higher concentration of H_2O_2 to kill PC12 cells than HT22 cells, suggesting that the antioxidant defense system in PC12 is much more effective than that of HT22.

Finally, five other toxins with proposed ROS intermediates in their toxicity pathways were examined: arsenite, cisplatin, nitric oxide (NO), MPP^+ and rotenone. Cisplatin and arsenite damage cells at least in part via oxidative damage to DNA (Hannemann and Baumann 1988). Both resistant cell lines are much more resistant to arsenite, while there is no significant difference with respect to cisplatin. Rotenone and MPP^+ are mitochondrial poisons and can cause Parkinson's disease-like symptoms when administered to animals (for review, see Andersen 2001). Both resistant cell lines are more resistant to rotenone, but only PC12r8 is slightly more resistant to MPP^+. Finally, NO has been implicated in many forms of cell death associated with CNS trauma and neurological disease (for review, see Bredt 1999). Cells were exposed to the NO donor diethylenetriamine/nitric oxide adduct, using diethylenetriamine as a control. There is no difference however, between cells which were selected for resistance to glutamate and Aβ and their parental cell lines regarding NO toxicity (Table 1). It follows that the regulation of oxidative damage caused by NO is very distinct from those of the other pro-oxidants.

The above data show that resistance to glutamate toxicity confers resistance to Aβ toxicity and *vice versa*, as well as resistance to several other neurotoxins. A related question is whether or not components of a defined programmed cell death pathway are shared in the toxicity of other toxins toward a single nerve cell type. To answer this question we killed HT22 hippocampal nerve cells with several neurotoxins and conditions which induce oxidative stress, and asked if these cell death pathways are blocked by reagents which inhibit a form of programmed cell death called oxytosis (Tan *et al.* 2002). Oxytosis is quite distinct from apoptosis in that the Bcl-2/Bax system is not involved (Dargusch *et al.* 2000), there is no DNA laddering and there are a number of morphological differences from apoptosis (Tan *et al.* 1998). A group of reagents have been identified which inhibit this pathway. These include the caspase inhibitor tyrosine-valine-alanine-aspartate-fluoromethyketone (YVADfmk), nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor (*R,S*)-3,5-dihydroxyphenylglycine (DHPG), a group I glutamate metabotropic receptor agonist, PD168,077, a dopamine D4 receptor agonist, the dietary antioxidant curcumin, diphenyleneiodium (DPI), a monoamine oxidase and mitochondrial ROS inhibitor, and finally, LY83583, a competitive inhibitor of soluble guanylate cyclase (sGC) (Tan *et al.* 2002). Table 2 shows the results when HT22 cells were challenged with glutamate, BSO, H_2O_2, the deprivation of exogenous cystine and glucose, NO, rotenone, sodium arsenite or cisplatin in the presence of these oxytosis inhibitors. Since cells can be overwhelmed by excess toxin, frequently masking some aspects of the cell death pathway, concentrations of toxins were chosen which only kill

Table 2 Protection of HT22 Cells from Various Toxins (EC50)

Inhibitor	Glutamate	Δcys*	BSO	H_2O_2	Arsenite	Cisplatin	NO	Rotenone
YVADfmk	1.5 μM	1.0 μM	3 μM	11 μM	No	No	No	No
NDGA	150 μM	160 μM	200 nM	270 nM	No	No	No	No
DHPG	‡	160 μM	No	No	No	No	120 μM	No
PD168, 077	35 μM	27 μM	8 μM	40 μM	No	No	No	60 μM
Curcumin	2 μM	4 μM	3 μM	15 μM	No	No	No	20 μM
DPI	5 nM	7 nM	No	No	No	No	No	No
LY83583	50 nM	45 nM	70 nM	30 nM	No	100 nM	500 nM	100 nM

Exponentially dividing HT22 cells were plated in microtiter plates at 2.5×10^3 cells per well and one day later challenged with the various toxins or toxic conditions at concentrations which killed between 60 and 80% of the cells as defined by the MTT assay 20 h later (see Table 1 for approximate concentrations in each toxic condition). Thirty minutes before adding the toxins the various inhibitors were added in triplicate at 6 different concentrations around their estimated EC50 as determined previously from glutamate toxicity. If no inhibition was observed, 6 higher concentrations were tested, or until the inhibitor itself became toxic. The data are presented as the concentration of the inhibitor that protected 50% of the cells from cell death. 'No' indicates that there was no protection at concentrations of up to 100 fold greater than those required to protect 50% of the cells from glutamate toxicity. *Δcys indicates the medium contains 65 μM cystine, 25% of the normal amount. ‡DHPG is a metabotropic glutamate receptor agonist whose biologic effect (protection) cannot be seen in the presence of high extracellular glutamate (see Sagara and Schubert 1998; for details).

between 60 and 80% of the cells. Cell death caused by glutamate toxicity and cystine deprivation were both blocked by all of the inhibitors of the oxytosis pathway. This is to be expected since glutamate is toxic by virtue of its ability to inhibit cystine uptake (Bannai and Kitamura 1980; Murphy *et al.* 1989).

The depletion of intracellular cystine uptake leads to the loss of intracellular GSH, the cell's major antioxidant. Since the loss of GSH is thought to initiate oxytosis, it would be expected that all reagents that inhibit glutamate toxicity would also block BSO-induced cell death. This is, however, not the case, for DPI and the metabotropic glutamate receptor agonist DHPG do not prevent BSO induced cell death (Table 2). DPI and DHPG are also ineffective against H_2O_2 toxicity. Since DPI blocks ROS production from mitochondria, which appears to be a necessary intermediate in oxytosis, it is not clear why BSO toxicity is unaffected. Since oxytosis involves the production of peroxides up to 300-fold above basal level, it would be expected that blocking mandatory cell death enzymes downstream of peroxide production would inhibit cell death caused by the addition of exogenous H_2O_2. This appears to be in part the case, for both PD168, 077 and LY83583, two reagents that inhibit Ca^{2+} influx, also block H_2O_2 toxicity (Li *et al.* 1997; Ishige *et al.* 2001). The activation of metabotropic glutamate receptors by DHPG does not block H_2O_2 toxicity, but the pathway used by DHPG to inhibit cell death is not well defined (Sagara and Schubert 1998).

In contrast to glutamate, BSO, H_2O_2 and cystine deprivation, the toxic insults of arsenite, cisplatin, rotenone, and NO were not inhibited by most of the reagents that block oxytosis. The major exception was the sGC inhibitor LY83583, which showed the widest spectrum of inhibition, blocking cell death induced by all of the toxic agents tested except arsenite. In addition, both PD168, 077 and the antioxidant, curcumin, blocked rotenone toxicity. DHPG was the only other inhibitor of NO toxicity besides the guanylate cyclase inhibitor. Therefore the specific cell death pathways which are activated by NO, rotenone, arsenite and cisplatin are distinct from the oxytosis pathway despite the fact that cells selected for resistance to oxytosis are generally more resistant to these toxins (Table 1).

Discussion

The above data show that nerve cells which are selected for resistance to Aβ or high extracellular glutamic acid are also more resistant to a variety of neurotoxins, including arsenite, rotenone, MPP^+ and the strong oxidizing agent, H_2O_2. While glutamate, BSO, cystine depletion, and H_2O_2 toxicities all share most of the cell death components required for oxytosis, cell death caused by arsenite, cisplatin, MPP^+, NO and rotenone all seem to occur by different mechanisms because most of the inhibitors that block oxytosis fail to inhibit the cell death caused by these toxins. These observations are somewhat surprising since the latter group of toxins are all thought to cause cell death via pathways which are ROS dependent, and the Aβ and glutamate resistant cells all have elevated antioxidant enzyme levels and are much more resistant to peroxides (Sagara *et al.* 1996; Sagara *et al.* 1998; Table 1). Therefore there must be multiple cell death pathways for these neurotoxins, and simple ROS elevation is apparently not always sufficient to complete the death program. The following paragraphs briefly discuss the cross-resistance and the putative role of ROS in cell death caused by each toxin.

There is rather extensive cross-resistance between cells selected in the presence of Aβ or glutamate and a variety of additional neurotoxins (Table 1). The shared resistance to cystine deprivation and BSO is not surprising since the loss of cystine and GSH are central to the oxytosis pathway (Tan *et al.* 2002). The pleotropic effects of cells selected for resistance to H_2O_2 has also been noted. For example, fibroblasts selected for resistance to H_2O_2 become resistant to cadmium(II) and mercury(II) (Sugiyama *et al.* 1993) as well as C2 ceramide (Kim *et al.* 2001) and exposure to pure oxygen (Spitz *et al.* 1995). Conversely, cells selected for resistance to $NiCl_2$ or cadmium also become resistant to H_2O_2 and menadione (Mello-Filho *et al.* 1988; Salnikow *et al.* 1994). Therefore cross-tolerance to a variety of toxic agents is not unusual and the common denominator appears to be resistance to oxidative stress that can be assayed by the cellular response to H_2O_2. It does not necessarily follow, however, that changes in antioxidant metabolism, although frequently observed, are the sole mechanism that leads to resistance (see above references and those that follow).

Table 1 shows that both the Aβ and glutamate resistant cells are not significantly more resistant to cisplatin than their parental cell lines. Cisplatin is generally considered a DNA damaging agent, causing DNA-DNA cross-linking as well as single strand breaks. There is evidence, however, that oxidative stress and resultant lipid peroxidation also contribute to the cytotoxicity caused by cisplatin (Hannemann and Baumann 1988; Spitz *et al.* 1993). H_2O_2-resistant HA1 fibroblasts are 1.5–3 fold more resistant to cisplatin than the parental lines (Spitz *et al.* 1993). It was shown, however, that the elevated catalase activity in the H_2O_2 and cisplatin resistant lines was not responsible for conferring resistance, and that elevated GSH was only in part responsible for cisplatin resistance (Spitz *et al.* 1993). These data are consistent with the fact that both Aβ-resistant PC12 cells (Sagara *et al.* 1996) and glutamate resistant HT22 cells (Sagara *et al.* 1998) have highly elevated antioxidant enzymes but are not resistant to cisplatin. It is therefore likely that although cisplatin can cause oxidative damage, its major mechanism of toxicity is independent of ROS metabolism.

Arsenic occurs naturally in soil, water, and air, and also as a by-product in the production of other metals. It has been

used for centuries as both a therapeutic and as an intentional poison; it is also a potent carcinogen (Abernathy *et al.* 1999). Rodent cells exposed to arsenite (As^{+3}), become cross resistant to arsenate (As^{+5}) (Gurr *et al.* 1999), as well as other metals such as cadmium and nickel (Romach *et al.* 2000), at least in part through the increase of multidrug resistant transporters which reduce intracellular metalloid concentration (Romach *et al.* 2000; Liu *et al.* 2001). Table 1 shows that cells selected for resistance to two agents which induce oxidative stress in cells, Aβ and glutamate, are also much more resistant to arsenite. These data suggest that at least some of the toxic effects of arsenite may be due to ROS metabolism. The major metabolite of inorganic arsenic, dimethylarsinic acid, causes the oxidation of deoxyguanosine to 8-oxo-2′-deoxyguanosine, probably through a dimethylarsenic peroxy radical (Yamanaka *et al.* 2001). Arsenite also directly causes the production of hydroxyl radicals and other ROS (Wang *et al.* 2001). Since both Aβ and glutamate resistant cells have elevated levels of enzymes which are able to reduce ROS toxicity, our data support the previous work on the involvement of ROS in arsenic induced mutagenicity and toxicity (Gurr *et al.* 1999).

Exposure of neurons to the Parkinson's inducing drugs 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), its metabolite MPP^+, or rotenone causes selective degeneration of dopaminergic neurons of the substantia nigra in mice and the death of HT22 and PC12 nerve-like cells (Table 1 and Andersen 2001; for recent review). Both rotenone and MPP^+ interfere with complex I mitochondrial electron transport, decrease ATP production, and increase mitochondrial generated ROS. Oxytosis also results in a 100–300-fold increase in mitochondria ROS production from complex I, which is required for the activation of down stream enzymes such as soluble guanylate cyclase (Tan *et al.* 2002). Surprisingly, the Aβ resistant cell lines were more resistant to MPP^+ and rotenone, but the glutamate resistant cells were only more resistant to rotenone.

Glutamate and cystine depletion kill cells by a well studied programmed cell death pathway called oxytosis (Tan *et al.* 2002). The death of HT22 cells and primary neurons caused by high extracellular glutamate is clearly inhibited by a number of reagents that interfere with defined steps in the pathway. Each step is necessary for cell death to occur. To determine if there are any shared steps in the pathways used by the various toxins, it was asked if the inhibitors of the oxytosis pathway also inhibit cell death caused by the other toxins. With the exception of cystine deprivation, BSO, and H_2O_2, all potent inducers of oxidative stress, the answer is generally no (Table 2). For example, NO, rotenone, cisplatin and arsenite toxicity are, with a few exceptions, not blocked by any inhibitor of oxytosis. The major exception is the ability of the sGC inhibitor, LY83583, to inhibit all toxicities except arsenite. sGC generates cGMP which, in turn, opens cGMP gated calcium channels, allowing the influx of extracellular calcium (Li *et al.* 1997; Ishige *et al.* 2001). If cyclase inhibitors such as LY83583 prevent this calcium flux, then the calcium does not enter and the cells do not die. The antioxidant curcumin and the dopamine D4 receptor agonist PD168, 077 also inhibit rotenone toxicity. It can be concluded from the combined data that although there is extensive overlap with respect to resistance to multiple neurotoxins, the cell death pathways employed by arsenic and cisplatin must be distinct from that causing oxytosis. Whether each of these neurotoxins employs a unique cell death pathway remains to be determined.

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